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BIOLOGY DIVISION
ANNUAL PROGRESS REPORT
For Period Ending December 31, 1967

R. F. Kimball, Director
S. F. Carson, Deputy Director

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JUNE 1968

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ORNL-1297	Period Ending May 10, 1952
ORNL-1393	Period Ending August 10, 1952
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ORNL-3427	Period Ending February 15, 1963
ORNL-3498	Period Ending August 15, 1963
ORNL-3601	Period Ending February 15, 1964
ORNL-3700	Period Ending August 15, 1964
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ORNL-3853	Period Ending July 31, 1965
ORNL-3922	Period Ending January 31, 1966
ORNL-3999	Period Ending July 31, 1966
ORNL-4100	Period Ending January 31, 1967

Contents

PUBLICATIONS AND LECTURES 1

Publications 1
Lectures 16
Visiting Lecturers 50
University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences – Biology Division Special Lecture Series 56
Scheduled Speakers at Professional Meetings in 1968 56

FOREIGN TRAVEL 58

MEETINGS AND CONFERENCES 63

1967 –
Proceedings of Twentieth Annual Biology Research Conference 63
Second International Conference on Cell Synchrony 64
AEC-NCI Cocarcinogenesis Review 64
Third Conference on Blood Platelets 65
Eighth Annual Symposium of the International Society for Cell Biology 65
Centrifugal Techniques for Vaccine Purification 65
Viral, Tumor, and Transplantation Antigen Isolation 66
Habrobracon-Mormoniella Conference 66
Annual Information Meeting 66
Symposium on Basic Mechanisms in Photochemistry and Photobiology 67
Meeting of Cancer Institute Directors 68
1967 Biomedical Program Directors Meetings 68
1967 Bone Marrow Conferences 68

1968 –
Tentative Program: Twenty-First Annual Biology Research Conference 70
International Symposium on Nuclear Physiology and Differentiation 72
1968 Bone Marrow Conferences 72

EDUCATIONAL ACTIVITIES 72

Student Trainee Program 72
Other Students 73
Senior Trainees 74
UT-Oak Ridge Graduate School of Biomedical Sciences 75
Graduate Courses Taught 75
Lectures Given in Other Courses, Institutes, and Special Programs 76
Special Courses Taken 77

INTERNATIONAL ACTIVITIES 78

DIVISION MEMBERS WORKING IN OTHER LABORATORIES 79

Returned from Laboratories Abroad 79
To Continue Education 79
At the Atomic Energy Commission 79
Collaborative Research 80

1. CYTOLOGY AND GENETICS 81

1.1 Photoreactivation and Mutagenesis in *Paramecium*– **R. F. Kimball, E. G. Bailiff** 81

1.2 Tests for Toxic and Mutational Effects of Irradiated Sucrose in *Paramecium* – **R. F. Kimball, E. G. Bailiff** 82

1.3 Chromosomal Damage Induced by Irradiated, Autoclaved Sucrose Solutions – **James D. Regan** 83

1.4 UV-Induced Pyrimidine Dimers in *Tetrahymena*. I. Excision – **G. L. Whitson, A. A. Francis, W. L. Carrier** 84

1.5 UV-Induced Pyrimidine Dimers in *Tetrahymena*. II. Photoreactivation – **G. L. Whitson, A. A. Francis** 84

1.6 UV Action Spectrum of Survival and Formation of Thymine-Containing Pyrimidine Dimers in *Tetrahymena* – **A. A. Francis, G. L. Whitson** 85

1.7 The Isolation of DNA and Mitochondria from *Tetrahymena* – **G. L. Whitson, A. A. Francis, W. D. Fisher** 86

1.8 Isolation and Characterization of UV-Sensitive Mutants of *Hemophilus influenzae* – **Jane K. Setlow, Dianne C. Brown, M. E. Boling, Alice A. Mattingly** 87

1.9 The Mechanism of Repair of UV Damage in *Hemophilus influenzae* – **M. E. Boling, S. P. Modak, M. P. Gordon, Alice A. Mattingly, Jane K. Setlow** 87

1.10 The Effect of a DNA Repair Mechanism in *Hemophilus influenzae* on the Photoreactivable Sector of Transforming DNA – **M. P. Gordon, Jane K. Setlow** 88

1.11 The Mechanism of Repair of X-Ray Damage in *Hemophilus influenzae* – **Gerald Price, Jane K. Setlow** 89

1.12 Defects in Transformation in *Hemophilus influenzae* – **Gerald Price, Jane K. Setlow** 89

1.13 The Effect of Acriflavine on the Sensitivity of UV-Irradiated Transforming DNA – **Jane K. Setlow, M. E. Boling** 90

1.14 Integration of Pyrimidine Dimers in Transforming DNA into the Recipient *Hemophilus influenzae* Cell Genome – **Alice A. Mattingly, Jane K. Setlow** 90

1.15 The Repair of UV Damage in Wild Type and Radiation-Sensitive Mutants of *Micrococcus radiodurans* – **B. E. B. Moseley** 91

1.16 On the Rejoining of Chromosome Breaks Induced by Fractionated X-Ray Exposures – **E. H. Y. Chu, Patricia C. Laaby** 91

1.17 Irradiation of Human Peripheral Blood in Vitro – **Karin E. Buckton, E. H. Y. Chu** 92

1.18 Chromosome Preparations from Mouse Peripheral Blood Cultured in Vitro and in Vivo – **Karin E. Buckton, Paul Nettesheim** 94

1.19 The Kinetics of X-Ray-Induced Chromatid Terminal Deletions and Gaps in Chinese Hamster Cells – **Sandra L. Bell, E. H. Y. Chu** 94

1.20 The Development and Applications of Vapor-Fixation Method for Vertebrate Chromosome Preparations – **Tatsuo Omura** 95

1.21 Possible Modification of Genetic Expression of Mammalian Cells with Monovalent Salts – **E. Virginia Merriam** 96

1.22 Isoantibody Separation – **Diana M. Popp, R. A. Brown** 96

1.23 Designation of Histocompatibility-14 Locus in the Mouse – **Diana M. Popp, George D. Snell** 98

1.24 Primary Structures of the α Chains of Hemoglobins from C3H and C3H·B Mice – **K. M. Hilse, J. E. Attrill, R. A. Popp, W. S. McEwen** 98

1.25 Transplantation Antigens of Mouse Spleen Cells – **R. A. Popp, N. G. Anderson, Diana M. Popp, W. S. McEwen** 100

1.26 Major Developmental Events in the *Rhynchosciara* Life Cycle – **Ellen Mattingly, C. L. Parker** 101

1.27 Spermatogenesis in Larval *Rhynchosciara sp.* – **C. L. Parker, Ellen Mattingly** 101

1.28 Varying Effects of X Radiation During the Life Cycle of *Rhynchosciara angelae* – **Ellen Mattingly** 102

1.29 Variations in Nucleolar Activity and RNA Synthesis During Development in *Rhynchosciara* – **Ellen Mattingly, Ronald Eckhardt** 102

1.30 Heterozygous Puffs and Bands in *Sciara ocellaris* Comstock (1882) – **A. L. P. Perondini, Crodowaldo Pavan** 103

1.31 Model Nucleoproteins – **D. E. Olins** 103

2. FUNGAL GENETICS 105

2.1 Dose-Rate Effects on the Spectrum of Recessive Lethal Mutations at Specific Loci in *Neurospora crassa* – **F. J. de Serres, H. V. Malling, B. B. Webber** 105

2.2 The Influence of Anoxia on X-Ray-Induced Recessive Lethal Mutation in *Neurospora crassa* – **F. J. de Serres** 107

2.3 Identification of the Genetic Alteration in Spontaneous Mutants at the *ad-3A* and *ad-3B* Loci of *Neurospora crassa* – **H. V. Malling, F. J. de Serres** 108

3. DROSOPHILA CYTOLOGY AND GENETICS 110

3.1 Distributive Pairing of Compound Autosomal Chromosomes in Females of *Drosophila melanogaster* – **E. H. Grell** 110

3.2 Mutants Lacking α-Glycerophosphate Dehydrogenase Activity in *D. melanogaster* – **E. H. Grell** 111

3.3 Response of Alcohol Dehydrogenase Levels to Doses of its Structural Gene in *Drosophila melanogaster* – **E. H. Grell** 111

3.4 The Genetic Variations of *Drosophila melanogaster* – **D. L. Lindsley, I. Lucile Norton, E. H. Grell, Elizabeth S. Von Halle** 112

3.5 Studies of Recognition at Exchange Pairing – **Rhoda F. Grell** 112

3.6 A Comparison of Chromosome Behavior at Exchange Pairing and at Distributive Pairing – **Rhoda F. Grell** 114

3.7 DNA Replication and Exchange in the *Drosophila* Oocyte – **Rhoda F. Grell, J. W. Day** 115

3.8 Nonhomologous Pairing in the Oogonia of *D. melanogaster* – **Rhoda F. Grell, J. W. Day** 117

3.9 Demonstration of Female Fertility of Homozygous Male-Sterile *X*-Autosome Translocations – **D. L. Lindsley, Mary S. Uziel** 117

3.10 Autosomal Nondisjunction in Meiotic Mutants of *Drosophila melanogaster* – **D. L. Lindsley, Mary S. Uziel** 118

3.11 DNA Measurements on Heterochromatin – **J. R. Merriam, Stella W. Perdue, R. F. Kimball** 119

3.12 Chromosome Pairing and Fertilization in Male *Drosophila melanogaster* – **J. R. Merriam** 120

3.13 On the Sequence of Elements in Compound-*XY* No. 129-16 and in Some of its Derivative Marked *Y* Chromosomes – **D. R. Parker** 121

3.14 Some Disjunctional Consequences of Translocation Induction in *Drosophila* Oocytes – **D. R. Parker** 122

3.15 Recovery of Multiple-Break Rearrangements from Irradiated Oocytes – **J. H. Williamson, D. R. Parker** 124

3.16 Interchromosomal Effects on Detachment of Attached-*X* Chromosomes in *D. melanogaster* – **J. H. Williamson** 124

3.17 Identification of *Y* Fragments with Two Doses of y^+ Or B^S – **J. H. Williamson** 125

3.18 EMS-Induced Sterile *Y* Chromosomes in *Drosophila melanogaster* – **J. H. Williamson** 126

4. YEAST, HYMENOPTERA, AND PHAGE GENETICS 127

4.1 The Enhanced Spontaneous Mutation Rate of an X-Radiation-Sensitive Yeast – **R. C. von Borstel, Kathleen J. La Brot, Dale E. Graham, Michael A. Resnick** 127

4.2 Dominant Lethal Mutations and Temperature-Sensitive Recessive Lethal Mutations Induced by Ethyl Methanesulfonate in *Habrobracon* Sperm – **Roger H. Smith** 128

4.3 Electrophoretic Variants of Xanthine Dehydrogenase in Geographical Races of *Habrobracon* – **Roger H. Smith, Kathryn Murray** 129

4.4 Cytochemical Localization of Sites of RNA Synthesis Using RNA Polymerase – **R. C. von Borstel, Joan W. Reel, S. P. Modak, F. J. Bollum** 130

4.5 Interference in Circular Genetic Maps – **C. M. Steinberg, Franklin W. Stahl** 132

5. CELLULAR RADIOBIOLOGY AND INTERNATIONAL ACTIVITIES 133

6. RADIATION MICROBIOLOGY AND MICROBIAL GENETICS 135

6.1 Control of Cell Division in *Escherichia coli* – **H. I. Adler, Alice A. Hardigree** 135

6.2 The Isolation of DNA from Minicells After Mating with F^+ Cells – **Amikam Cohen, W. D. Fisher, H. I. Adler** 136

6.3 Giant Cells of *Escherichia coli* – **H. I. Adler, C. E. Terry, Alice A. Hardigree** 136

6.4 The Influence of an Episome on the Sensitivity of *Escherichia coli* K-12 to Ionizing Radiation – **D. E. Axelrod, H. I. Adler** 137

6.5 Time-Lapse Cinematography of Nuclear Division in Bacteria – **H. I. Adler, Alice A. Hardigree** 137

6.6 Conjugal Transfer of F′ Factors of *Escherichia coli* into *Proteus mirabilis* – **G. J. Stine, Annie S. Angel, Roy Curtiss III** 138

6.7 Bacterial Conjugation with Hfr Donors of *Escherichia coli* K-12 – **Roy Curtiss III** 138

6.8 Bacterial Conjugation with F^+ Donors of *Escherichia coli* K-12 – **Roy Curtiss III, D. R. Stallions** 140

7. HUMAN CYTOGENETICS 142

7.1 Statistical Analysis of the Human Karyotype – **M. A Bender, M. A. Kastenbaum** 142

7.2 Photoreactivation Among the Classes of Vertebrates – **James D. Regan, John S. Cook, W. H. Lee** 145

7.3 The Excision of Pyrimidine Dimers from the DNA of Ultraviolet-Irradiated Human Cells – **James D. Regan, W. L. Carrier, J. E. Trosko** 145

7.4 The Effect of Tritiated Thymidine on Sister Chromatid Exchange in a Ring Chromosome – **J. G. Brewen, W. J. Peacock** 147

7.5 Chromosome Aberrations and the Exchange Hypothesis – **J. G. Brewen, R. D. Brock** 148

8. MAMMALIAN GENETICS 150

8.1 The Frequencies of X-Chromosome Loss in Female Mice Exposed to Doses of 50 and 400 r of X Rays – **W. L. Russell, Elizabeth M. Kelly, Patricia R. Hunsicker, Carolyn M. Vaughan** 151

8.2 Comparative Study of Five X-Autosome Translocations Involving LG1 in the Mouse – **L. B. Russell, Clyde S. Montgomery** 152

8.3 RNA Synthesis in Mouse Oocytes – **E. F. Oakberg, Evelyn C. Lorenz, Roselynn A. Dynesius, Diane W. Slover** 154

8.4 Stem-Cell Renewal in the Seminiferous Epithelium of the Mouse – **E. F. Oakberg, Evelyn C. Lorenz, Roselynn A. Dynesius, Diane W. Slover** 154

8.5 Transmission of Radiation-Induced Dominant Skeletal Mutations in Mice – **U. H. Ehling** 155

8.6 Toxicity, Fertility, and Dominant Lethal Mutations After Injection of Irradiated Sucrose in Mice – **U. H. Ehling, D. G. Doherty, Dorma J. Gottlieb, Sandra W. Huff** 156

8.7 ^{3}H-Uridine Incorporation into LM Cells in Vivo and in Vitro and into Normal Mouse Tissues – **R. B. Cumming** 157

9. MAMMALIAN RECOVERY 160

9.1 Mortality from Secondary Disease in Radiation Chimeras – **C. C Congdon, R. E. Toya, Sr.** 160

9.2 Kidney Lysozyme in Hypoxic Radiation Chimeras – **Eiji Endo** 162

9.3 Morphologic Basis for Liver Enlargement – **W. H. McArthur** 163

9.4 Karyo-Interkaryometry in Liver of Radiation Chimeras – **W. H. McArthur** 164

9.5 Early Proliferation of Transplanted Spleen Colony-Forming Cells – **A. L. Kretchmar, W. R. Conover** 164

9.6 Short-Term Bone Marrow Cultures – **L. H. Smith, T. W. McKinley, Jr.** 166

9.7 Size of the Hemopoietic Compartment as a Factor in Radiosensitivity of the Mouse – **L. H. Smith, T. W. McKinley, Jr.** 167

9.8 Influence of Thymus Cells on Erythropoiesis of Parental Marrow in Irradiated Hybrid Mice – **Joan Wright Goodman, Sarah G. Shinpock** 168

9.9 Modification of Growth of Parental Marrow in Posttreated F_1 Hybrid Mice – **Joan Wright Goodman, H. Brownell Wheeler** 170

9.10 A Method of Closed Gas Anesthesia for Laboratory Animals – **M. L. Simmons, L. H. Smith** 172

9.11 Anesthesia in Laboratory Rats – **M. L. Simmons, J. B. Jones** 172

9.12 Replication of a Minute DNA Animal Virus – **R. W. Tennant, K. R. Layman, R. E. Hand, Jr.** 174

9.13 Incidence of Murine Virus Antibody in Humans in Contact with Laboratory Animals – **R. W. Tennant, Roberta K. Reynolds, K. R. Layman** 176

9.14 Observations on Normobaric Hypoxia Encephalopathy in Mice – **C. B. Richter, R. E. Toya, Sr.** 177

9.15 Some Normal Blood Values for Mice – **W. J. Miller, C. B. Richter** 180

10. RADIATION IMMUNOLOGY 182

10.1 Ribonucleic Acid Synthesis of Phytohemagglutinin-Transformed Lymphocytes in Vitro – **Hiroo Yamamoto, Takashi Makinodan, R. A. Brown** 182

10.2 Time-Dependent Cytological Studies of Antibody-Forming Cells Exposed to 10,000 r of X Rays – **Toshihiko Sado** 183

10.3 The Enumeration of Antibody-Forming Cells in Lymphoid Tissue Following Antigen Stimulation – **Glenda C. Webb, E. H. Perkins** 185

10.4 Growth and Differentiation of Murine Myeloma Cells in Diffusion Chamber Culture – **Dona C. Hooper, J. F. Albright** 186

10.5 A Low-Molecular-Weight Antibody – **R. A. Brown, J. M. Ellis** 187

10.6 Toxic Factors in the Spleens of Aging Mice and Prolongation of Life by Splenectomy – **J. F. Albright, Julia W. Deitchman, Takashi Makinodan** 187

10.7 Assessment of Hemolytic Plaque-Forming Cells in Millipore Diffusion Chambers – **D. L. Groves** 188

10.8 The Regulation of Antibody Production – **D. C. Vann** 189

10.9 Evidence for Synthesis of Low-Molecular-Weight Incomplete Antibody by Spleen Cells of Aged Mice – **Takashi Makinodan, R. A. Brown, E. H. Perkins, W. J. Peterson, J. M. Ellis, Shirley C. Tipton** 190

10.10 Further Characterization of a Cell Transfer System for the Study of Antigen-Sensitive Progenitor Cells – **M. J. Bosma, E. H. Perkins, Takashi Makinodan** 191

11. GENERAL PHYSIOLOGY 193

11.1 Control of Cell Division in *E. coli* Filaments – **W. D. Fisher, F. W. Shull, Jr., H. I. Adler, Amikam Cohen** 193

12. CELL PHYSIOLOGY 195

12.1 Survival of Supercooled Yeast – **Peter Mazur, R. H. Miller** 195

12.2 Nucleation of Supercooled Solutions of Native and Denatured Macromolecules – **Peter Mazur, R. H. Miller** 197

12.3 Effect of Freezing on the Sensitivity of Cells to Toxic Chemicals – **Steven Henikoff, Peter Mazur, S. P. Leibo** 197

12.4 Freezing of Bacteriophage T4B – **S. P. Leibo, Peter Mazur** 199

12.5 Observations on *os* Mutants of Bacteriophage T4 – **S. P. Leibo, Edouard Kellenberger, Peter Mazur, C. M. Steinberg** 200

13. CHEMICAL PROTECTION AND ENZYME CATALYSIS 201

13.1 Chemical Protection – **D. G. Doherty, Margaret A. Turner, Frances E. Hacker** 201

13.2 Studies on the Substrate Specificity of Pepsin – **D. G. Doherty, Jesse James, Margaret A. Turner** 202

14. PATHOLOGY AND PHYSIOLOGY 204

14.1 Low (Medium) Level Experiment – **All Members of Pathology-Physiology Section** 205

14.2 Preliminary Observations on the Modification of Late Somatic Effects of Radiation by Hypoxia – **E. B. Darden, Jr., M. L. Davis, M. C. Jernigan, K. W. Christenberry, G. E. Cosgrove, J. W. Conklin** 206

14.3 Evolution of Chromosome Aberrations in Radiation-Induced and Passaged Mouse Granulocytic Leukemia – **Niel Wald, A. C. Upton, S. Pan, R. C. Brown** 207

14.4 Responsiveness of Leukemic Cells to Growth-Regulating Factors – **A. C. Upton, Lou C. Satterfield** 207

14.5 Hemopoiesis in Irradiated RFM Mice as Studied by the Spleen Colony Assay – **V. K. Jenkins, A. C. Upton, T. T. Odell, Jr.** 208

14.6 Effect of Estradiol on Hemopoiesis in RFM Mice – **V. K. Jenkins, A. C. Upton, T. T. Odell, Jr.** 210

14.7 Influence of Age on Hemopoietic Repopulation of the Spleen After Whole-Body Irradiation in RFM Mice – **M. L. Davis, A. C. Upton, G. E. Cosgrove** 210

14.8 X-Ray and X-Ray-Plus-Urethane Induction of "Leukemogenic" Activity in Tissues of Mice: Electron Microscopic Studies – **Vincenzo Covelli, R. C. Brown, R. L. Tyndall** 212

14.9 Ultrastructural Studies of a High-Mortality Lapine Enteritis – **R. C. Brown, C. B. Richter, M. D. Bloomer** 213

14.10 Cytogenetic Studies of Hemophilia-Like Disease of Swine – **R. C. Brown, C. N. Cornell, K. W. Cole, K. M. Brinkhous** 214

14.11 The Development of a Reliable Gram Stain for Bacteria in Tissue Sections – **R. C. Brown, Howard C. Hopps** 214

14.12 Generation Cycle of Rat Megakaryocytes – **T. T. Odell, Jr., C. W. Jackson, Rebecca S. Reiter** 215

14.13 Analysis of Microspectrophotometric Measurements of DNA of Megakaryocytes – **D. G. Gosslee, T. T. Odell, Jr., C. W. Jackson** 216

14.14 Size Distribution of Blood Platelets and Erythrocytes for 74 Days After X Irradiation – **T. T. Odell, Jr., R. S. Reiter, C. W. Jackson** 216

14.15 Influence of Microbial Flora and X Radiation on Reticular Neoplasms of RFM Mice – **H. E. Walburg, Jr., G. E. Cosgrove** 217

14.16 Foreign Spleen Reaction in Sublethally X-Irradiated Germfree F_1 Hybrid Mice – **H. E. Walburg, Jr., G. E. Cosgrove** 218

14.17 ^{125}I-Labeled Iododeoxyuridine Incorporation as a Measure of Hemopoietic Recovery in X-Irradiated Mice – **H. E. Walburg, Jr., Edna I. Mynatt, E. A. Wulfsberg** 219

14.18 Concerning the Specificity of Tumor Inhibition Resulting from Immunization with Rauscher Leukemia Virus Vaccine – **R. L. Tyndall, J. A. Otten, N. K. Clapp** 220

14.19 Further Purification of Mouse Plasma Erythropoietin and the Effect of Its Presence on Plasma Esterase Isoenzymes – **R. C. Allen, D. J. Moore, J. W. Fisher** 221

14.20 Erythropoietin Inhibitor in Kidney Extracts and Plasma from Anemic Uremic Human Subjects – **J. W. Fisher, B. L. Roh, F. E. Hatch, R. C. Allen, B. J. Kelley** 221

14.21 Possible Use of Enzyme Markers to Follow the Genetic Stability of Donor and Host Cells in Mouse Radiation Chimeras – **R. C. Hard, Jr., R. C. Allen** 222

15. CELL GROWTH AND DIFFERENTIATION 223

15.1 Sequential Changes in the Sedimentation Profile of RNA Synthesized During Tissue Transformation – **D. H. Reese, Egidio Puccia, Tuneo Yamada** 223

15.2 Culture of Iris Cells from *Triturus viridescens* – **D. H. Reese** 225

15.3 Localization of Labeled Actinomycin D in Differentiating Lens Regenerate Cells – **Tuneo Yamada, Marion E. Roesel** 225

15.4 Availability of Intraperitoneally Injected Tritiated Thymidine for Labeling Lens Regenerate Cells Transplanted in the Newt Optic Chamber – **Tuneo Yamada, Marion E. Roesel** 226

15.5 Histological and Cytological Patterns of Incorporation of Tritiated Uridine into Lens Regenerates Cultured in Vitro – **Tuneo Yamada, Marion E. Roesel** 227

15.6 Immunofluorescence Studies on the Lens-Specific Proteins in Amphibian Lens Development – **D. S. McDevitt, Isaura Meza** 227

15.7 Immuno-Electron Microscopic Studies on the Localization of γ Crystallins in Amphibian Lens Cells – **Rolf Nöthiger, D. S. McDevitt, Isaura Meza** 228

15.8 Preparation of Specific Antibodies Against γ Crystallins of the Newt Triturus Viridescens – **D. S. McDevitt, Rolf Nöthiger** 229

15.9 Studies on Terminal Cell Differentiation in Chick Lens. I. Analysis of Nuclear Degeneration, Ploidy, and DNA Loss – **S. P. Modak, Stella W. Perdue, R. F. Kimball** 230

15.10 Studies on Terminal Cell Differentiation in Chick Lens. II. Analysis of Priming Ability and Denaturability of DNA from Various Cell Populations of Differentiating Lens Using Calf Thymus DNA Polymerase – **S. P. Modak, R. C. von Borstel, F. J. Bollum** 232

15.11 Studies on Terminal Cell Differentiation in Chick Lens. III. Analysis of the Priming Ability of DNA from Various Lens Cell Populations Using *Micrococcus* RNA Polymerase – **S. P. Modak, R. C. von Borstel, F. J. Bollum** 234

15.12 Observations on Isolated Components of Amphibian Oocyte Nucleoli – **O. L. Miller, Jr., Barbara R. Beatty** 235

15.13 Synaptinemal Complexes in Meiotic Chromosomes – **George Gassner III** 238

15.14 Aspects of Vitellogenesis in the South African Clawed Toad, *Xenopus laevis* – **R. A. Wallace, D. W. Jared, Diana Rudack** 238

15.15 Cytologic and Histochemical Observations of Oocyte Maturation in the Annelid *Enchytraeus albidus* – **J. N. Dumont** 239

15.16 The Origin and Formation of Cortical Granules in the Oocytes of Amphioxus, *Branchiostoma caribaeum* – **J. N. Dumont** 241

15.17 Cytologic Changes Associated with Spermiogenesis in *Rhynchosciara sp.* – **J. N. Dumont, Ronald Eckhardt, Ellen Mattingly** 241

15.18 Isolation of Hemocyanin from Hemolymph of *Callinectes sapidus* – **Marilyn S. Kerr** 242

15.19 Incorporation of Leucine into Hemocyanin During Short-Term Organ Culture of Hemocytes and Hepatopancreas from the Blue Crab – **Marilyn S. Kerr** 243

16. BIOCHEMISTRY OF CELL DIFFERENTIATION 245

16.1 The Synthesis of Lens Proteins by Epithelial Cells in Tissue Culture – **John Papaconstantinou, J. W. Carr** 245

16.2 The Regulation of Ribosomal RNA Synthesis in Bovine Lens Epithelial Cells and Fiber Cells – **John Papaconstantinou, Emilia M. Julku** 247

16.3 An Increase in the Complexity of α-Crystallins During Lens Cell Differentiation – **Winifred G. Palmer, John Papaconstantinou** 248

16.4 An Immunofluorescent Analysis of the Distribution of α-Crystallins in Embryonic and Adult Bovine Lens Cells – **R. E. Shore, Winifred G. Palmer, John Papaconstantinou** 248

16.5 The Stabilization of Protein-Synthesizing Templates in Bovine Lens – **J. A. Stewart, John Papaconstantinou** 250

16.6 Studies on Ribosomal Degradation in Lens Cells – **W. F. Zapisek, John Papaconstantinou** 250

17. ENZYMOLOGY 252

17.1 Isoaccepting tRNA's in Mouse Plasma Cell Tumors – **Wen-Kuang Yang, G. David Novelli** 252

17.2 Transfer RNA's During Development in *Artemia salina* – **J. C. Bagshaw, F. J. Finamore, G. David Novelli** 253

17.3 Protein Synthesis of Isolated Polysomes – **Peter Pfuderer, Donna G. Shaffer** 254

17.4 Hydrolysis of Oligoribonucleotides by an Enzyme Fraction from *Escherichia coli* – **Audrey L. Stevens, S. K. Niyogi** 254

17.5 Studies of Complexes of RNA Polymerase from *E. coli* with Polyribonucleotides – **Audrey L. Stevens** 254

17.6 Pilot Plant Activities – **Mary V. Long, E. F. Phares** 255

17.7 The Interaction of Phenylalanyl-RNA Synthetase and $tRNA^{Phe}$ – **M. P. Stulberg, T. W. Martin, Jr., S. A. Fairfield, K. R. Isham, J. W. Longworth** 255

17.8 Studies on Multiple $tRNA^{His}$ in Histidine-Repressed Systems from *Bacillus subtilis* – **M. P. Stulberg, K. R. Isham, Audrey L. Stevens** 256

17.9 Isoenzymes: Their Regulation and Genetic Control – **K. Bruce Jacobson, J. A. Knopp, J. B. Murphy** 256

17.10 *E. coli* Valine tRNA Synthetase: Purification – **P. O. Ritter, K. Bruce Jacobson** 257

18. NUCLEIC ACID CHEMISTRY 258

18.1 tRNA Structure and Function – **Mayo Uziel, H. G. Gassen, A. Jeannine Bandy, Chongkun Koh** 258

18.2 A Specific Cleavage of tRNA Chains – **Mayo Uziel** 259

18.3 On the Use of TLC and TLE in the Separation and Characterization of Oligonucleotides – **H. G. Gassen** 260

18.4 The Properties of Pyrimidine Disulfides – **B. C. Pal** 260

18.5 Structural Similarities in Transfer RNA's – **Mayo Uziel, J. X. Khym, Chongkun Koh, A. Jeannine Bandy** 261

18.6 Purification of tRNA – **B. C. Pal** 262

19. NUCLEIC ACID ENZYMOLOGY 263

19.1 Biochemical Analysis of Naturally Rejoined Sections of DNA Strands of Bacteriophage T5 – **R. K. Fujimura, Elliot Volkin** 263

19.2 Purification of Bacteriophage T2 mRNA – **W. S. Riggsby, Elliot Volkin** 265

19.3 Competition in DNA-RNA Hybridization Experiments – **W. S. Riggsby, Virginia Merriam** 265

19.4 The Stability of Oligoadenylate-Polyuridylate Complexes as Measured by Thermal Chromatography – **S. K. Niyogi** 266

19.5 Studies on the Role of Chain Length and Base Composition in the Specific Association of Ribooligonucleotides with Denatured DNA – **S. K. Niyogi** 266

19.6 The Genetic Translational Apparatus of Mitochondria – **W. E. Barnett, L. S. Dure III, J. L. Epler, Diane J. Goins** 267

19.7 Coding Properties of *Neurospora* Mitochondrial and Cytoplasmic Transfer RNA's – **J. L. Epler, Diane J. Goins, W. E. Barnett, R. A. Dolbeer** 268

19.8 DNA Synthesis in *Artemia salina* – **C. G. Mead** 268

19.9 Nucleotide Sequence Analyses – **J. X. Khym, Mayo Uziel** 268

20. DEVELOPMENTAL BIOCHEMISTRY 270

20.1 Role of P^1,P^4-Diguanosine 5′-Tetraphosphate in the Synthesis of DNA by *Artemia salina* – **F. J. Finamore, Rose P. Feldman** 270

20.2 An Investigation of Trehalase Activity in *Artemia salina* – **R. D. Ewing** 271

21. BIOCHEMICAL REGULATION 272

21.1 Hormonal Regulation of Tyrosine Transaminase in Fetal Rat Liver in Organ Culture – **W. D. Wicks** 272

21.2 Hormone Effects on Hybridizable RNA from Rat Liver – **Claudio Pellegrino, F. T. Kenney** 273

21.3 Hormonal Control of Tyrosine Transaminase in Isolated, Perfused Rat Liver – **C. B. Hager, G. R. Holloway, F. T. Kenney** 274

21.4 Hormonal Control of RNA Transport – **C. B. Hager, F. T. Kenney** 274

21.5 Stimulation of Liver Ribonucleic Acid Synthesis by Estrogen – **J. L. Wittliff, F. T. Kenney, R. A. Wallace** 275

22. MICROBIOLOGY 276

22.1 Kinetics of Malic-Lactic Transhydrogenase – **M. I. Dolin** 276

23. PLANT PHYSIOLOGY AND PHOTOSYNTHESIS 277

23.1 Light Retardation of Functional and Ultrastructural Chloroplast Deterioration and Its Independence from Photosynthesis – **A. H. Haber, Paula J. Thompson, Patricia L. Walne, L. L. Triplett** 277

23.2 Canceling Errors in the Quantitative Determination of α-Amylase Activity – **A. H. Haber, L. L. Triplett** 278

23.3 Immediate and Delayed Effects of Lethal Doses of Gamma Radiation on Aspects of Photosynthesis and on Ultrastructure of Chloroplasts and Other Organelles – **P. L. Walne, A. H. Haber, W. A. Arnold, J. R. Azzi** 279

23.4 Induced Mitotic Activity and Disorganized Growth in Relation to Auxin-Induced Tumors – **D. E. Foard, A. H. Haber, Helen J. Luippold, Rhonda G. Farmer** 280

23.5 Radiation-Induced Cell Lethality and Shoot Meristem Collapse in the Absence of Mitosis – **D. E. Foard, A. H. Haber, Sarah A. Angel, Pat C. Kerr** 281

23.6 Using Colchicine to Determine the Number of Cells Initially Forming a Lateral Root Primordium – **D. H. O'Dell, D. E. Foard, Rhonda G. Farmer** 282

23.7 Photosynthesis – **W. A. Arnold, W. F. Bertsch, J. B. Davidson, J. R. Azzi, R. M. Pearlstein** 283

24. MICROBIAL PHOTOSYNTHESIS 285

24.1 The Molecular Diversity of the Primary Photosynthetic Carboxylation Enzyme – **Louise E. Anderson, G. B. Price, Jr., R. C. Fuller** 285

24.2 Pteridines in Photosynthesis – **N. A. Nugent, R. C. Fuller** 286

24.3 Regulation of Activity of Calvin Cycle Enzymes in *Rhodospirillum rubrum* – **Louise E. Anderson, R. C. Fuller** 287

25. BIOPHYSICS 288

25.1 Chromosome Replication in *Escherichia coli* – **L. G. Caro, Claire M. Berg** 288

25.2 The Incorporation of 5-Bromouracil in the Chromosome of *Escherichia coli* K12 – **L. G. Caro, Claire M. Berg, D. P. Allison** 289

25.3 Excision of λdg Prophage from the Bacterial Chromosome – **R. A. Weisberg** 290

25.4 Study of Transduction by Phage P1 – **Grete Kellenberger-Gujer** 291

25.5 Some Properties of Isolated Proplastids from Etiolated Maize Leaves – **Ann B. Jacobson, R. W. Williams** 292

25.6 Sedimentation Properties of Ribosomal RNA from Proplastids of Maize – **Ann B. Jacobson, R. W. Williams** 293

25.7 The Selective Loss of DNA Satellites on Deproteinization with Phenol – **Dorothy M. Skinner** 294

25.8 Studies on the Biological Role of a DNA Satellite – **Dorothy M. Skinner** 294

25.9 Determination of the Molecular Weight of Mitochondrial DNA in *Physarum polycephalum* – **R. A. McGrath, R. W. Williams** 295

25.10 Use of Chemical ^{3}H-Methylation in the Determination of Sedimentation Coefficients of DNA in Alkaline Sucrose – **J. S. Cook, A. J. Zelnis** 295

25.11 Photoreactivation and Photoreactivating Enzyme in Eukaryotic Cells – **J. S. Cook, Janet R. McGrath** 296

25.12 The Existence of Cyclobutane Pyrimidine Dimers in DNA – **R. B. Setlow, W. L. Carrier** 296

25.13 The Excision of Pyrimidine Dimers in Vivo and in Vitro – **R. B. Setlow, W. L. Carrier** 297

25.14 Photoreactivation in Vivo of the DNA of T4 Phage – **R. B. Setlow, W. L. Carrier** 297

25.15 Pyrimidine Dimers in Paramecium DNA – **Betsy M. Sutherland, W. L. Carrier, R. B. Setlow** 298

25.16 Photoreactivation, Photoprotection, and Photoinactivation of Induced Formation of Enzymes in *Escherichia coli* B/r – **P. A. Swenson** 299

25.17 Inhibition of β-Galactosidase Synthesis by UV in Escherichia Coli B in the Absence of Catabolite Repression – **P. A. Swenson** 299

25.18 The Physiology of UV-Irradiated *Escherichia coli* – **Barbara A. Hamkalo, P. A. Swenson** 300

25.19 ATP Levels in UV-Irradiated *Escherichia coli* B/r – **Barbara A. Hamkalo, P. A. Swenson, J. W. Longworth** 300

25.20 Evidence for Two Kinds of Base Stacking in Poly rA – **R. O. Rahn, J. W. Longworth, R. I. Fox** 301

25.21 Aggregation of Single-Stranded DNA with Mg^{2+} – **R. O. Rahn** 301

25.22 Influence of Base Stacking on Thymine Dimer Formation in DNA: Solvent and Temperature Perturbations – **R. O. Rahn, J. L. Hosszu** 302

25.23 Low-Temperature Photochemistry and Photobiology of DNA – **R. O. Rahn, Jane K. Setlow, J. L. Hosszu** 302

25.24 Photochemistry and Photobiology of Bacterial Spores: Temperature Dependence – **J. E. Donnellan, Jr., J. L. Hosszu, R. O. Rahn, R. S. Stafford** 303

25.25 The Ultraviolet Photochemistry of Germinating Spores – **R. S. Stafford, J. E. Donnellan, Jr.** 303

25.26 Some Stereochemical Features of Thymidylyl Dimers in a Polynucleotide – **J. W. Longworth** 305

25.27 Energy Transfer in Poly-L-Tyrosine at 77°K – **J. J. ten Bosch, R. O. Rahn, J. W. Longworth** 305

25.28 Specific Gamma-Ray Emission Coefficient of ^{137}Cs-^{137m}Ba – **M. L. Randolph** 306

25.29 Is Age-Dependent Genetic Damage in Seeds Caused by Free Radicals? – **A. D. Conger, M. L. Randolph** 307

26. COOPERATIVE PROGRAMS 309

AEC-NCI Cocarcinogenesis 309

26.1 Concerning the Use of Viruses to Transmit Genetic Information – **Stanfield Rogers** 310

26.2 The Isolation of Poly-Lysine Induced by Poly-Adenine-Modified Tobacco Mosaic Virus RNA in Tobacco Plants – **Stanfield Rogers, Peter Pfuderer** 311

26.3 Ethionine Carcinogenesis – **Beryl Ortwerth, G. David Novelli** 312

26.4 Ethylation of Hepatic tRNA by Ethionine in Vivo – **Lawrence Rosen, Shigemi Iiyama, G. David Novelli** 312

26.5 Biochemical Studies in Rat Liver During Ethionine Carcinogenesis – **Ugo Del Monte, G. David Novelli** 313

26.6 tRNA's in Rat Spleens During Immune Response – **Wen-Kuang Yang, G. David Novelli, C. J. Wust** 314

26.7 tRNA's of L-M Cells in in Vitro Culture and in in Vivo Tumor – **Wen-Kuang Yang, D. H. Martin, Alfred Hellman, Kiki B. Hellman, G. David Novelli** 314

26.8 Isolation of Ribosomal RNA from Rat Spleen – **C. J. Wust, D. G. Ridner** 315

26.9 Ribosomes of Rat Spleen – **C. J. Wust, D. G. Ridner** 315

26.10 Studies on the Purification and Characterization of tRNA Pyrophosphorylase – **Audrey N. Best, G. David Novelli** 316

26.11 Pulmonary and Hepatic Oncogenesis After Dimethylnitrosamine (DMN) Treatment of Male RF Mice – **N. K. Clapp, A. W. Craig, R. E. Toya, Sr.** 317

26.12 Diethylnitrosamine Carcinogenesis in BALB/c Mice – **N. K. Clapp, R. L. Tyndall, J. A. Otten** 318

26.13 Altered Esterase Zymograms Associated with Leukemia Virus Infection – **R. L. Tyndall, R. C. Allen, Dorothy J. Moore, Ernestine Teeter** 319

26.14 Mammalian Cell Genetics. I. Selection and Characterization of Mutations – **E. H. Y. Chu, E. Virginia Merriam, Patricia A. Brimer** 319

26.15 Mammalian Cell Genetics. II. Chemical Mutagenesis – **E. H. Y. Chu, H. V. Malling** 321

26.16 Posttreatment by Streptomycin and Mutation by MNNG in *Paramecium* – **R. F. Kimball, E. G. Bailiff, Stella W. Perdue** 322

26.17 Chemical Modification of Proteins – **F. C. Hartman** 323

26.18 Instrumentation for X-Ray Diffraction – **J. R. Einstein** 323

26.19 Structure of a Photodimer of 1-Methylthymine – **J. R. Einstein, C. H. Wei** 323

26.20 Preparation and Preliminary X-Ray Data on Thymine Dimer Potassium Salt – **C. H. Wei** 324

26.21 X-Ray Crystal Structure Determinations of Two Transition-Metal Complexes – **C. H. Wei** 324

26.22 Singlet and Triplet Energy Transfers in L-Tryptophanyl-L-Tyrosine and Related Heteropolypeptides – **J. W. Longworth, R. O. Rahn, J. A. Knopp** 325

26.23 Singlet Energy Transfer in Ribonuclease – **J. W. Longworth, Maria Del Carmen Battista, J. A. Knopp** 326

26.24 Energy Transfer in Poly-L-Tyrosine: Fluorescence Polarization Studies – **J. A. Knopp, J. W. Longworth** 327

26.25 Exciplex Formation in Model Systems and Proteins – **J. A. Knopp, J. W. Longworth** 328

26.26 Further Tales of Tyrosine – **Maria Del Carmen Battista, J. W. Longworth** 328

26.27 Genetic Analysis of Hydroxylamine-Induced Purple Mutants (*ad-3*) in *Neurospora crassa* – **H. V. Malling, F. J. de Serres** 328

26.28 The Photodynamic Effect of Carcinogenic Polycyclic Hydrocarbons in *Neurospora crassa* and Chinese Hamster Cells – **H. V. Malling, E. H. Y. Chu, F. J. de Serres** 331

26.29 Development of Selective Systems for Somatic Cell Hybridization – **James D. Regan, Susumu Takeda, W. H. Lee** 332

26.30 Synchronization of Lower-Vertebrate Cells by the Antineoplastic Agent Hydroxyurea – **Susumu Takeda, James D. Regan** 333

26.31 Inhalation Carcinogenesis – **D. G. Doherty, Alfred Hellman, J. O. Brick, Paul Nettesheim, M. G. Hanna, Jr., R. F. Newell** 335

26.32 Computer Programs Used in the Inhalation Carcinogenesis Project – **M. G. Hanna, Jr., J. O. Brick, B. S. Bishop** 338

26.33 Serum Alpha Globulin Fraction: Survival-and-Recovery Effect in Irradiated Mice – **M. G. Hanna, Jr., Paul Nettesheim, W. D. Fisher** 339

26.34 Localization of ^{125}I-Labeled Antigen in Germinal Centers of Mouse Spleen: Effects of Competitive Injection of Specific or Non-Crossreacting Antigen – **M. G. Hanna, Jr., Mary W. Francis** 341

26.35 The Ultrastructure of Antigen Localization and Virus-Like Particles in Mouse Spleen Germinal Centers – **A. K. Szakal, M. G. Hanna, Jr.** 343

26.36 Effect of X Irradiation on the Localization and Persistence of Antigen – **Paul Nettesheim, M. G. Hanna, Jr.** 346

26.37 Inhalation Co-Carcinogenesis Barrier Facility – **J. O. Brick** 346

26.38 Fate of 1-(4-Dimethylaminobenzylidene)indene in Rats – **C. T. Bahner, D. G. Doherty** 347

26.39 Studies on in Vivo Protein Photooxidation by Monitoring Tritium Loss from in Vivo Histidine Moieties – **A. P. Harrison, Jr.** 347

26.40 Regulation of Enzyme Synthesis and Turnover – **F. T. Kenney, Joseph Kendrick, L. E. Roberson** 348

26.41 Regulation of Enzyme Synthesis and Changes in Transfer RNA's During Diethylnitrosamine Carcinogenesis – **F. T. Kenney, Joseph Kendrick, Lawrence Rosen, L. E. Roberson** 349

26.42 Early and Late Effects of Hydrocortisone on the Synthesis of Tyrosine Transaminase in Rat Liver – **J. L. Wittliff, F. T. Kenney** 349

26.43 Induction of Glucokinase in Cultured Reuber H-35 Hepatoma – **J. R. Reel, F. T. Kenney** 349

26.44 Regulation of Tyrosine Transaminase Synthesis by Hydrocortisone in Hepatoma Cell Cultures – **J. R. Reel, F. T. Kenney** 350

26.45 Hormonal Regulation of Mitochondrial Enzymes from Hepatoma Cell Cultures – **J. L. Wittliff, F. T. Kenney** 351

AEC-NIGMS 352

26.46 Differential Induction of Dominant Lethal Mutations in Mice by Various Alkyl Methanesulfonates – **U. H. Ehling, D. G. Doherty, H. V. Malling** 352

26.47 Induction of Specific Locus Mutations by Alkylating Agents in Male Mice – **U. H. Ehling, R. B. Cumming, H. V. Malling** 354

26.48 Chemical Induction of Dominant Lethals in Female Mice – **W. M. Generoso** 355

26.49 Strain Variation in the Sensitivity of Mice to Alkylating Agents – **W. M. Generoso** 358

26.50 Changes in *E. coli* Leucine tRNA After Bacteriophage T2 Infection – **L. C. Waters, G. David Novelli** 360

26.51 Isolation of Two Formylmethionine Transfer RNA's from *Escherichia coli* by Reversed-Phase Chromatography – **L. R. Shugart, A. D. Kelmers, Barbara Chastain, G. David Novelli** 361

26.52 Restoration of Aminoacylation Activity of Undermethylated tRNA by in Vitro Methylation – **L. R. Shugart, Barbara Chastain, M. P. Stulberg** 361

26.53 Studies on the Phenylalanine tRNA Synthetases of *Neurospora crassa*. Purification – **F. J. Kull, K. Bruce Jacobson** 362

AEC-NASA Space Biology 363

26.54 Acute Effects of 62-Mev Protons in Mice – **J. L. Montour, E. B. Darden, Jr., N. K. Clapp, M. C. Jernigan, A. C. Upton** 363

26.55 Further Observations on the RBE of 60-Mev Protons for Late Somatic Effects in Mice – **E. B. Darden, Jr., N. K. Clapp, K. W. Christenberry, A. C. Upton, M. C. Jernigan, J. W. Conklin, J. J. Beauchamp** 364

26.56 Mutational Response of *Habrobracon* in the Biosatellite II Experiment – **R. C. von Borstel, A. C. Hoffman, Roger H. Smith, Martha S. Jones, D. S. Grosch, Sohei Kondo, Anna R. Whiting, Margaret J. Lane, R. L. Amy, T. J. Mizianty, M. B. Baird, Mary Lou Pardue, P. Buchanan, Joan W. Reel, Katherine T. Cain, Diana B. Smith, Ruth Ann Carpenter, Judith A. Steen, A. M. Clark, Julie T. Tindall, L. R. Valcovic** 365

26.57 Shifts in the Recessive Lethal Mutation Spectrum in *Habrobracon* – **Roger H. Smith, Anna R. Whiting, R. C. von Borstel** 368

26.58 The *Neurospora* Experiment (P-1037) on Biosatellite II – **F. J. de Serres, B. B. Webber** 368

Publications and Lectures

PUBLICATIONS

During the period February 1 through December 31, 1967, 501 manuscripts written or edited by members of the Biology Division for publication in the open literature were published or sent to press; in addition 12 ORNL reports were issued.

Listed below are the 179 papers, 3 issues of *Experimental Hematology*, 1 coedited book, the *Symposium on Chromosome Mechanics at the Molecular Level*, and 12 ORNL reports published during the period. Not listed are the 74 abstracts and 2 book reviews published, or the 241 items currently in press.

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Adler, H. I.	Discussion III: bacteriology	In, *Proceedings of the Conference on Radiation Biology Held at the Oak Ridge Associated Universities, Oak Ridge, Tennessee, August 2–5, 1965* (Sponsored by ORNL, UT Agricultural Research Laboratory, and ORAU in cooperation with the USAEC), USAEC Division of Technical Information Extension, Oak Ridge, Tenn., 1967, pp. 59–61
Adler, H. I., W. D. Fisher, Amikam Cohen, and Alice A. Hardigree	Miniature *Escherichia coli* cells deficient in DNA	*Proc. Natl. Acad. Sci. U.S.* **57**, 321–26 (1967)
Anderson, N. G. (Program Coordinator)	The Molecular Anatomy of Cells and Tissues (the MAN Program) – annual report for the period July 1, 1965 to June 30, 1966	ORNL-3978
	The Molecular Anatomy of Cells and Tissues (the MAN Program) – annual report for the period July 1, 1966 to June 30, 1967	ORNL-4171
Anderson, N. G.	Preparative zonal centrifugation	In *Methods of Biochemical Analysis*, vol. 15 (ed. by David Glick), Interscience Publishers, Division of John Wiley & Sons, New York, London, Sydney, 1967, pp. 271–310
Anderson, N. G., and G. B. Cline	Semiannual contract progress report to the National Institute of Allergy and Infectious Diseases, March 1 to July 1, 1966	ORNL-4024

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Anderson, N. G., G. B. Cline, W. W. Harris, and J. G. Green	Isolation of viral particles from large fluid volumes	In, *Transmission of Viruses by the Water Route* (ed. by G. Berg), Interscience Publishers, Division of John Wiley & Sons, New York, London, Sydney, 1967, pp. 75–88
Anderson, N. G., and J. G. Green	The soluble phase of the cell	In, *Enzyme Cytology* (ed. by D. B. Roodyn), Academic Press, London and New York, 1967, pp. 475–509
Anderson, N. G., and Elizabeth Rutenberg	Analytical techniques for cell fractions. VII. A simple gradient-forming apparatus	*Anal. Biochem.* **21**, 259–65 (1967)
Anderson, N. G., D. A. Waters, W. D. Fisher, G. B. Cline, C. E. Nunley, L. H. Elrod, and C. T. Rankin, Jr.	Analytical techniques for cell fractions. V. Characteristics of the B-XIV and B-XV zonal centrifuge rotors	*Anal. Biochem.* **21**, 235–52 (1967)
Barnett, W. E., and D. H. Brown	Mitochondrial transfer ribonucleic acids	*Proc. Natl. Acad. Sci. U.S.* **57**, 452–58 (1967)
Barnett, W. E., D. H. Brown, and J. L. Epler	Mitochondrial-specific aminoacyl-RNA synthetases	*Proc. Natl. Acad. Sci. U.S.* **57**, 1775–81 (1967)
Barnett, W. E., and J. L. Epler	Multiple aminoacyl-RNA synthetase systems and the genetic code in Neurospora	*Cold Spring Harbor Symp. Quant. Biol.* **31**, 549–55 (1966)
Bender, M. A	Synergistic effect of zero-*g* and radiation on white blood cells and *Neurospora* spores – annual report, period ending June 30, 1966	ORNL-TM-1715
Bender, M. A, J. B. Davidson, and M. A. Kastenbaum	Chromosome analysis	In, *Use of Computers in Analysis of Experimental Data and the Control of Nuclear Facilities*, AEC Symposium Series 10, B. I. Spinrad (Coordinator), U.S. Atomic Energy Commission/Division of Technical Information Extension, Oak Ridge, Tenn., 1967, pp. 121–32
Bender, M. A, F. J. de Serres, P. Carolyn Gooch, Ida Ruth Miller, D. B. Smith, and Sohei Kondo	Experiment S004, radiation and zero-G effects on human blood and *Neurospora*	In, *Interim Report: Manned Space Flight Experiments – Gemini XI Mission, September 12–15, 1966*, MSC-TA-R-67-2, Science and Applications Directorate, Manned Spacecraft Center, Houston, Tex., May 1967, pp. 45–66
Bender, M. A, and P. Carolyn Gooch	Chromosome aberrations in irradiated humans	In, *Progress in Radiology*, vol. II (ed. by L. Turano, A. Ratti, and C. Biagini, and coeditors E. H. Burrows, M. Pellier, and H. R. Schinz), International Congress Series No. 105, Excerpta Medica Foundation, Amsterdam, New York, London, Milan, Tokyo, Buenos Aires, 1967, pp. 1421–25
	Synergistic effect of zero-G and radiation on white blood cells and Neurospora spores	ORNL-TM-1995

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Bender, M. A, P. Carolyn Gooch, and Sohei Kondo	The Gemini-3 S-4 spaceflight-radiation interaction experiment	*Radiation Res.* **31**, 91–111 (1967)
Bennett, Michael, and Gustavo Cudkowicz	Functional and morphological characterization of stem cells: The unipotential role of "lymphocytes" of mouse marrow	In, *The Lymphocyte in Immunology and Haemopoiesis – Symposium Held at Bristol, April 1966* (ed. by J. M. Yoffey), Edward Arnold (Publishers) Ltd., London, 1967, pp. 183–94
Berg, Claire M., and L. G. Caro	Chromosome replication in *Escherichia coli*. I. Lack of influence of the integrated F factor	*J. Mol. Biol.* **29**, 419–31 (1967).
Berg, Claire M., and Roy Curtiss III	Transposition derivatives of an Hfr strain of *Escherichia coli* K-12	*Genetics* **56**, 503–25 (1967)
Bertsch, W. F., J. R. Azzi, and J. B. Davidson	Delayed light studies on photosynthetic energy conversion. I. Identification of the oxygen-evolving photoreaction as the delayed light emitter in mutants of *Scenedesmus obliquus*	*Biochim. Biophys. Acta* **143**, 129–43 (1967)
Boling, M. E., and Jane K. Setlow	Photoreactivating enzyme in logarithmic-phase and stationary-phase yeast cells	*Biochim. Biophys. Acta* **145**, 502–5 (1967)
Bollum, F. J.	Biosynthetic polydeoxynucleotides	In, *Procedures in Nucleic Acid Research* (ed. by G. L. Cantoni and D. R. Davies), Harper & Row Publishers, New York and London, 1966, pp. 577–83
	Filter paper disk techniques for assaying radioactive macromolecules	*Ibid.*, pp. 296–300
	Preparation of deoxynucleotide polymerizing enzymes from calf thymus gland	*Ibid.*, pp. 284–95
	Preparation of oligodeoxynucleotides	*Ibid.*, pp. 592–99
	Ribonucleic acid – structure and function	In, *Encyclopedia of Biochemistry* (ed. by R. J. Williams and E. M. Lansford, Jr.), Reinhold Publishing Corporation (subsidiary of Chapman-Reinhold, Inc.), New York, Amsterdam, London, 1967, pp. 734–35
Bosma, M. J., Takashi Makinodan, and H. E. Walburg, Jr.	Development of immunologic competence in germfree and conventional mice	*J. Immunol.* **99**, 420–30 (1967)
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Swartzendruber, D. C.	Observations on the ultrastructure of lymphatic tissue germinal centers	In, *Germinal Centers in Immune Responses* (ed. by H. Cottier, N. Odartchenko, R. Schindler, and C. C Congdon), Springer-Verlag New York, 1967, pp. 71–76
Tennant, R. W., R. K. Reynolds, and K. R. Layman	Incidence of murine virus antibody in humans in contact with experimental animals	*Exptl. Hematol.* **14**, 76 (1967)
Terry, C. E., B. J. Kilbey, and H. B. Howe, Jr.	The nature of photoreactivation in *Neurospora crassa*	*Radiation Res.* **30**, 739–47 (1967)
Terry, C. E., and Jane K. Setlow	Photoreactivating enzyme from *Neurospora crassa*	*Photochem. Photobiol.* **6**, 799–803 (1967)
Trosko, J. E., and J. G. Brewen	Cytological observations on the strandedness of mammalian metaphase chromosomes	*Cytologia* **31**, 208–12 (1966)
	Inhibition of ultraviolet-induced chromosome breaks by cysteamine in 5-bromouracil-substituted mammalian cells	*Radiation Res.* **32**, 200–13 (1967)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Trosko, J. E., and Margaret R. Kasschau	Study of pyrimidine dimers in mammalian cells surviving low doses of ultraviolet radiation	*Photochem. Photobiol.* **6**, 215–19 (1967)
Tyndall, R. L., and R. C. Allen	Altered esterase zymograms associated with infection with leukaemia virus	*Nature* **215**, 984–85 (1967)
Tyndall, R. L., J. A. Otten, Ernestine Teeter, and N. D. Bowles	Inhibition of solid tumor formation by prior immunization with formalized neoplastic spleen extracts	*Proc. Soc. Exptl. Biol. Med.* **125**, 399–402 (1967)
Tyndall, R. L., and Ernestine Teeter	Observations on the RNA metabolism of leukaemic cell cultures	*Nature* **215**, 79–80 (1967)
Upton, A. C.	Radiation effects in mammalian tissues	In, *Proceedings of the Conference on Radiation Biology Held at the Oak Ridge Associated Universities, Oak Ridge, Tennessee, August 2–5, 1965* (Sponsored by ORNL, UT Agricultural Research Laboratory, and ORAU in cooperation with the USAEC), USAEC Division of Technical Information Extension, Oak Ridge, Tenn., 1967, pp. 25–35
	Retiring President's report to the Radiation Research Society for the year 1965–66	*Radiation Res.* **31**, 174–77 (1967)
Upton, A. C., J. W. Conklin, G. E. Cosgrove, W. D. Gude, and E. B. Darden	Necrotizing polyarteritis in aging RF mice	*Lab. Invest.* **16**, 483–87 (1967)
Upton, A. C., J. W. Conklin, and R. A. Popp	Influence of age at irradiation on susceptibility to radiation-induced life-shortening in RF mice	In, *Radiation & Ageing, Proceedings of a Colloquium Held in Semmering, Austria, June 23–24, 1966* (ed. by Patricia J. Lindop and G. A. Sacher), Taylor & Francis Ltd., London, 1966, pp. 337–44
Uziel, Mayo	Desalting of nucleotides by gel filtration	In, *Methods in Enzymology* (ed. by S. P. Colowick and N. O. Kaplan); vol. XII, *Nucleic Acids*, Part A (ed. by L. Grossman and K. Moldave), Academic Press, New York and London, 1967, pp. 407–14
Vazquez, J. J., and Takashi Makinodan	Cytokinetic events following antigenic stimulation	*Federation Proc.* **25**, 1727–33 (1966)
von Borstel, R. C.	Summary progress report to the National Aeronautics and Space Administration, February 17, 1964–June 30, 1966	ORNL-TM-1731
	Quarterly progress report to the National Aeronautics and Space Administration, July 1 to September 30, 1966	ORNL-TM-1732

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
	Quarterly progress report to the National Aeronautics and Space Administration, October 1–December 31, 1966	ORNL-TM-1957
	Quarterly progress report to the National Aeronautics and Space Administration, January 1–March 31, 1967	ORNL-TM-1958
Walburg, H. E., Jr., and G. E. Cosgrove	Ageing in irradiated and unirradiated germfree ICR mice	*Exptl. Gerontol.* **2**, 143–58 (1967)
Walburg, H. E., Jr., G. E. Cosgrove, and A. C. Upton	Longevity as influenced by irradiation in germfree mice	In, *Radiation & Ageing, Proceedings of a Colloquium Held in Semmering, Austria, June 23–24, 1966* (ed. by Patricia J. Lindop and G. A. Sacher), Taylor & Francis Ltd., London, 1966, pp. 361–65
Wallace, R. A., Susan L. Walker, and P. V. Hauschka	Crustacean lipovitellin. Isolation and characterization of the major high-density lipoprotein from the eggs of decapods	*Biochemistry* **6**, 1582–90 (1967)
Warner, A. H., and F. J. Finamore	Nucleotide metabolism during brine shrimp embryogenesis	*J. Biol. Chem.* **242**, 1933–37 (1967)
Waters, L. C., and G. David Novelli	A new change in leucine transfer RNA observed in *Escherichia coli* infected with bacteriophage T2	*Proc. Natl. Acad. Sci. U.S.* **57**, 979–85 (1967)
Wei, C. H., G. R. Wilkes, and L. F. Dahl	The crystal structure of twinned tetrarhodium dodecacarbonyl	*J. Am. Chem. Soc.* **89**, 4792–93 (1967)
Whiting, Anna R.	The biology of the parasitic wasp *Mormoniella vitripennis* (Walker)	*Quart. Rev. Biol.* **42**, 333–406 (1967)
Whitson, G. L., W. D. Fisher, G. M. Padilla, and I. L. Cameron	Cell synchrony (a review of the 2nd International Conference on Cell Synchrony)	*Science* **157**, 1219–24 (1967)
Whitson, G. L., J. G. Green, A. A. Francis, and D. D. Willis	Cyclic changes in the alcohol-soluble carbohydrates in synchronized *Tetrahymena*. I. Fractionation	*J. Cell Physiol.* **70**, 169–77 (1967)
Wicks, W. D.	RNA synthesis in fetal rat liver	*Arch. Biochem. Biophys.* **121**, 55–66 (1967)
Wolff, Sheldon, and F. J. de Serres	Chemistry of crossing-over	*Nature* **213**, 1091–92 (1967)
Wust, C. J.	Clearance of sheep and rat erythrocytes in the mouse: Selenium-75 as marker	*RES – J. Reticuloendothelial Soc.* **4**, 43–50 (1967)
	Studies on ribosomes of rat spleen during the immune response	*Arch. Biochem. Biophys.* **118**, 568–76 (1967)
Wust, C. J., and M. G. Hanna, Jr.	The effect of actinomycin D on the immune response to two antigens given in sequence	*RES – J. Reticuloendothelial Soc.* **3**, 415–23 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Yamada, Tuneo	Cellular and subcellular events in Wolffian lens regeneration	In, *Current Topics in Developmental Biology*, vol. 2 (ed. by A. Monroy and A. A. Moscona), Academic Press, New York, 1967, pp. 247–83
	Cellular synthetic activities in induction of tissue transformation	In, *Ciba Foundation Symposium: Cell Differentiation* (ed. by A. V. S. De Reuck and J. Knight), J. & A. Churchill, London, 1967, pp. 116–26
	Differentiation of lens cells	In, *Experimental Biology and Medicine*, vol. 1 – *Morphological and Biochemical Aspects of Cytodifferentiation* [ed. by E. Hagen, W. Wechsler, P. Zilliken, and (co-editor) A. F. Gardner], S. Karger, Basel and New York, 1967, pp. 77–89
	Factors of embryonic induction	In, *Comprehensive Biochemistry*, vol. 28 (ed. by M. Florkin and E. H. Stotz), Elsevier Publishing Company, Amsterdam, London, New York, 1967, pp. 113–43
Zalik, Sara Eisenberg, and Tuneo Yamada	The cell cycle during lens regeneration	*J. Exptl. Zool.* **165**, 385–93 (1967)

LECTURES

During the period February 1 through December 31, 1967, members of the Biology Division presented 517 lectures and speeches in the United States and abroad. Of these, 228 were given at professional society meetings; 253 at the invitation of universities, scientific organizations and institutions, and professional groups or clubs; and 36 under the Traveling Lecture Program.

For details on speeches outside the continental United States, see the section **Foreign Travel**; educational activities are described in the section of that name (see Contents).

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I.	Cell division mutants of *Escherichia coli*	Third Conference on Information and Control Processes in Living Systems (New York Academy of Sciences), Pacific Palisades, Calif.
	Genetic control of cell division in *Escherichia coli*	(1) Washington Area Radiobiological Association, Washington, D.C. (2) Yeshiva University, New York City

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I.	Genetic control of cell growth and division in bacteria	University of Chicago, Chicago
	Genetic control of cell growth and division in *Escherichia coli*	(1) Rockefeller University, New York City (2) Lawrence Radiation Laboratory, University of California, Berkeley (3) Georgetown University, Washington, D.C. (4) Division of Biology and Medicine, USAEC, Washington, D.C.
	Genetic control of radiation sensitivity and cell division in *Escherichia coli*	Thessaloniki Cancer Institute, Athens, Greece
	Radiation sensitivity of bacteria	Casaccia Nuclear Research Center, Rome, Italy
Adler, H. I. (Amikam Cohen, W. D. Fisher, and Alice A. Hardigree)	A new cell division mutant of *Escherichia coli* (*Bacteriol. Proc.*, 1967, p. 58)	American Society for Microbiology (67th Annual Meeting), New York City
Adler, H. I. (and Alice A. Hardigree)	Growth and division of *Escherichia coli*	Kentucky-Tennessee Branch of the American Society for Microbiology, Memphis
Allen, R. C.	Acrylamide gel electrophoretic separation of mouse plasma erythropoietin	Medical Unit of the University of Tennessee, Memphis
	Qualitative and quantitative changes in esterase isoenzymes and plasma and tissue of mice	Medical College of Virginia, Richmond
Allen, R. C. (Dorothy J. Moore, and J. W. Fisher)	Further purification of mouse plasma erythropoietin and the effect of its presence on plasma esterase isoenzymes	American Society of Hematology (10th Annual Meeting), Toronto, Ontario, Canada
Anderson, Louise	Light and metabolites; control of the Calvin cycle in *Rhodospirillum rubrum*	University of Illinois at Chicago Circle, Chicago
Anderson, N. G.	An approach to cell injury at the molecular level	University of Sussex, Brighton, England
	Centrifugal methods for large scale virus vaccine purification	Xth International Congress of the Permanent Section of Microbiological Standardization, Prague, Czechoslovakia

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Anderson, N. G.	Centrifugal systems for virus isolation and purification	Meeting on Centrifugal Techniques for Vaccine Purification, Oak Ridge, Tenn.
	Development of zonal centrifuges	Rockefeller Institute, New York City
	Instrumentation for biophysical research	NIH Conference on Future Goals for Engineering, Biology, and Medicine, Washington, D.C.
	Instrumentation and separation systems for the MAN program	1967 San Diego Biomedical Engineering Symposium, San Diego, Calif.
	Molecular anatomy	Conference on Science for Clergymen, ORAU, Oak Ridge, Tenn.
	The Molecular Anatomy Program	University of California, Los Angeles
	New zonal techniques for isolating liver cell membranes	Wistar Symposium on Biological Properties of the Mammalian Surface Membrane, Philadelphia
	Research on the liquid centrifuge	U.S. Army Nuclear Science Seminar (7th Annual), Oak Ridge, Tenn.
	Zonal centrifugation	Union Carbide Corporation, New York City
Anderson, N. G. (E. L. Candler, and L. H. Elrod)	Studies on adenovirus 31 hamster tumor antigen(s)	Viral, Tumor, and Transplantation Antigen Isolation Conference, Oak Ridge, Tenn.
Andrews, G. A.[1] (C. C Congdon, C. L. Edwards, Nazareth Gengozian, B. Nelson, and Helen Vodopick)	Preliminary trials of clinical immunotherapy	Conference on Acute Leukemia and Burkitt's Tumor, Rye, N.Y.
Arnold, W. A.	Photosynthesis	Cornell University, Ithaca, N.Y.
Axelrod, D. E.	Genetics and physiology of radiation resistant mutants of the bacterium *Escherichia coli*	Temple University, Philadelphia

[1]Medical Division, ORAU.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Axelrod, D. E. (and H. I. Adler)	Genetic factors that increase radiation resistance of *Escherichia coli* (*Bacteriol. Proc.*, 1967, p. 48)	American Society for Microbiology (67th Annual Meeting), New York City
	Radiation resistance conferred on *Escherichia coli* by the episomal fertility factor	Kentucky-Tennessee Branch of the American Society for Microbiology, Memphis
Baccarini, Iracema (T. T. Odell, Jr., A. C. Upton, and W. D. Gude)	An autoradiographic study of vaginal epithelium of mice by serial biopsy [*Anat. Record* **157**, 207–8 (1967)]	American Association of Anatomists, Kansas City, Mo.
Bagshaw, J. C.	Studies on transfer RNA's during development in *Artemia salina*	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Barnett, W. E.	Mitochondrial protein synthesis	(1) Louisiana State University, Baton Rouge (2) University of Texas, Austin (3) Purdue University, Lafayette, Ind. (4) Illinois State University, Normal
	Mitochondrial tRNAs and aminoacyl-RNA synthetases	(1) University of Tennessee, Knoxville (2) University of Georgia, Athens (2 lectures)
	The role of RNA in protein synthesis	Mary Hardin Baylor College, Belton, Tex.
	s-RNA specific ligases	Gerontology Branch, Baltimore City Hospitals, Baltimore
	Studies on the genetic code and gene enzyme relationships	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
Barnett, W. E. (D. H. Brown, G. D. Novelli, Sally Sherard, and J. L. Epler)	Mitochondrial transfer RNAs and aminoacyl-RNA synthetases	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Barnett, W. E. (J. L. Epler, and D. H. Brown)	Mitochondrial-specific tRNAs and aminoacyl-RNA synthetases [*Federation Proc.* **26**, 734 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Chicago

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Bender, M. A	Effects of radiation on chromosomes [2]	Symposium on the Peaceful Uses of Atomic Radiation, Rio de Janeiro, Brazil
	Gemini S-4 experiments	Space Science Seminar, Oak Ridge, Tenn.
Bertsch, W. F.	Delayed light measurements on photosynthetic energy conversion	(1) University of Chicago, Chicago (2) School of Biological Sciences, Northwestern University, Evanston, Ill.
Best, Audrey N. (and G. David Novelli)	Purification of and studies with ATP: tRNA adenylyltransferase from *Escherichia coli* (read by title)	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Brewen, J. G.	Autoradiographic studies on the nature and behaviour of chromosomal subunits	(1) Battelle Northwest Laboratories, Richland, Wash. (2) Michigan State University, East Lansing
	Chromosomes and space biology	Division of Plant Industry, CSIRO, Canberra, Australia
	(1) The evidence for a multistranded model of eukaryote chromosomes (2) The theory of chromosome aberrations	University of Adelaide, Adelaide, Australia
Brick, J. O. (R. F. Newell, and D. G. Doherty)	History of a rodent breeding and experimental colony housed in filter top cages	American Association for Laboratory Animal Science (18th Annual Meeting), Washington, D.C.
Brown, R. C. (and H. C. Hopps)	The development of a reliable gram stain for bacteria in tissue sections	VIth Latin American Congress of Pathology – Ist Pan American Meeting of the International Academy of Pathology, San Juan, Puerto Rico
Buckton, Karin E.	Irradiation-induced chromosome aberrations in humans	University of California, Los Angeles
	The types and frequencies of chromosome aberrations in peripheral blood following irradiation *in vivo*	New England Medical Center, Boston

[2]Paper presented for Dr. Bender by Dr. José Carlos Cabral de Almeida.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Caro, L. G.	Chairman's remarks	Medical Symposium on Radioisotopes in Medicine: *in vitro* Studies, ORAU, Oak Ridge, Tenn.
	Chromosome replication in *E. coli* K12	(1) University of Sussex, Brighton, England (2) Pasteur Institute, Paris, France (3) Laboratory of Genetics, Toulouse University, Toulouse, France
	Studies on chromosome replication in *E. coli*	(1) New York University School of Medicine, New York City (2) Rockefeller University, New York City
Carrier, W. L.	Biophysical studies on DNA – effects of ultraviolet irradiation	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
Choat, E. E.[3] (M. L. Simmons, L. P. Wynns, and E. E. Choat)	A facility design for pathogen-free, inbred mouse production [ASHRAE **9**, 27–31 (1967)]	(1) American Society of Heating, Refrigeration, and Air Conditioning Engineers, Knoxville, Tenn. (2) American Association for Contamination Control (6th Annual Technical Meeting), Washington, D.C.
Chu, E. H. Y.	Genetics of cultured mammalian cells	City of Hope Medical Center, Duarte, Calif.
	Genetic studies of mammalian somatic cells in culture	Temple University, Philadelphia
Chu, E. H. Y. (and Patricia Brimer)	Mutational changes in the resistance of Chinese hamster cells to 8-azaguanine [*Mammalian Chromosome Newsletter* **9**, 29 (1968)]	Conference on Mammalian Cytology and Somatic Cell Genetics (6th Annual), Pacific Grove, Calif.
Chu, E. H. Y. (E. Virginia Merriam, and Patricia Brimer)	Genetic studies of mammalian cells *in vitro* [*Genetics* **56**, 550 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.

[3]Y-12 Engineering.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Clapp, N. K.	Alkylating nitrosamines and carcinogenesis	U.S. Army Nuclear Science Seminar (7th Annual), Oak Ridge, Tenn.
	(1) Murine leukemias in the RF mouse (2) Oncogenic effects of diethylnitrosamine in the mouse	Colorado State University, Fort Collins
	Suggested interference in DEN-carcinogenesis by simultaneous administration of methyl methane sulfonate	Environmental Science Center, Research Triangle Park, N.C.
Clapp, N. K. (A. W. Craig, and A. C. Upton)	Comparative oncogenic effects of diethylnitrosamine, other alkylating agents, and radiation in mice [*Radiation Res.* **31**, 552 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Cline, G. B.	Continuous-flow-with-banding. Application to vaccine purification	Meeting on Centrifugal Techniques for Vaccine Purification, Oak Ridge, Tenn.
Cline, G. B. (Manuel Rosenbaum, and N. G. Anderson)	The isolation of viral soluble antigens by gradient resolubilization (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 120)	Biophysical Society (11th Annual Meeting), Houston, Tex.
Cohen, Amikam	Isolation of episomal DNA from minicells after mating with *E. coli* F^+ cells	University of Pittsburgh School of Medicine, Pittsburgh, Pa.
	The minicells of *E. coli* as a tool for investigating transfer of DNA during bacterial mating	Washington University School of Medicine, St. Louis
Cohen, Amikam (H. I. Adler, W. D. Fisher, and F. W. Shull)	Induction of cell division in *Escherichia coli* filaments (*Bacteriol. Proc.*, 1967, p. 58)	American Society for Microbiology (67th Annual Meeting), New York City
Cohen, Amikam (D. P. Allison, H. I. Adler, and Roy Curtiss III)	Genetic transfer to minicells of *Escherichia coli* K-12 [*Genetics* **56**, 550–51 (1967)]	Genetics Society of America, Stanford, Calif.
Congdon, C. C	(1) Biology Division animal research facilities (2) Secondary disease in radiation chimeras (3) Morphologic changes during an immune response	Oncological Institute, Bucharest, Romania
	General principles of pathology	University of Tennessee, Knoxville
	Lymphatic tissue pathology in radiation chimeras	Workshop on Immunology of Leprosy, Bethesda, Md.
	Radiation recovery	U.S. Army Nuclear Science Seminar (7th Annual), Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Congdon, C. C (T. J. Mitchell, D. A. Gardiner, M. A. Kastenbaum, and C. C Congdon)	Ninety day mortality in irradiated mice treated with rat bone marrow [*Federation Proc.* **26**, 571 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Exptl. Pathol.), Chicago
Congdon, C. C (M. L. Simmons, and R. E. Toya)	The effect of hypoxia on mortality from secondary disease [*Exptl. Hematol.* **13**, 32 (1967)]	Bone Marrow Conference, Chicago
	Reduced mortality from secondary disease after exposure to hypoxia	First International Congress of the Transplantation Society, Paris, France
Cook, Dorothy Skinner	Isolation and characterization of the satellite DNA's of the land crab, *Gecarcinus lateralis*	American Association for the Advancement of Science, New York City
	Satellite DNA's in the land crab *Gecarcinus lateralis*	American Society for Cell Biology, Denver, Colo.
Cook, J. S.	Direct demonstration that yeast photoreactivating enzyme and light monomerize thymine-containing dimers in UV-irradiated DNA (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 78)	Biophysical Society (11th Annual Meeting), Houston, Tex.
	The effects of ultraviolet radiation on cells and cellular repair mechanisms	U.S. Army Nuclear Science Seminar (7th Annual), Oak Ridge, Tenn.
	Photoreactivation	University of Tennessee, Knoxville
	The role of DNA in ultraviolet-induced mitotic delay in sand dollar zygotes	American Society for Cell Biology, Denver, Colo.
Cosgrove, G. E.	Delayed irradiated effects on laboratory mice	ORINS Medical Division Seminar, Oak Ridge, Tenn.
Cosgrove, G. E. (P. B. Dunaway, T. P. O'Farrell, J. A. Payne, and H. E. Childs, Jr.)	Pathology survey in small mammals [*Bull. Wildlife Diseases Assoc.* **3**, 117 (1967)]	Wildlife Disease Association, Urbana, Ill.
Cosgrove, G. E. (A. C. Upton, and C. C Congdon)	Effects of hemopoietic cell injection on the incidence of reticular tissue neoplasms in irradiated mice [*Radiation Res.* **31**, 554 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Cosgrove, G. E.	Delayed effects of low-level radiation	(1) ORAU Medical Radioisotopes Course, Oak Ridge, Tenn. (2) ORAU Health Physics Summer Institute, Oak Ridge, Tenn. (3) ORAU Health Physics Course, Oak Ridge, Tenn.
	(1) Parasites and parasitism (2) Snakes	Biology Club, Oak Ridge High School, Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Cudkowicz, Gustavo	Hybrid resistance to parental grafts of hematopoietic and lymphoma cells	XXIst Annual Symposium on Fundamental Cancer Research, Houston, Tex.
Curtiss, Roy, III	Bacterial conjugation	Michigan State University, East Lansing
	Mechanisms of chromosome mobilization and transfer during bacterial conjugation	Cold Spring Harbor Bacterial Genetics seminar, Cold Spring Harbor, N.Y.
	Mechanisms involved during bacterial conjugation in *Escherichia coli*	North Carolina State University, Raleigh
	(1) Quantitative analysis in virology (2) Radiobiology of bacterial viruses	University of Tennessee, Knoxville
Curtiss, Roy, III (D. R. Stallions, Jane A. Mays, and Janet Renshaw)	Mechanism of chromosome mobilization and transfer by F^+ donors of *Escherichia coli* K-12 [*Genetics* **56**, 553–54 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Curtiss, Roy, III (Jane A. Mays, and D. R. Stallions)	Macromolecular syntheses and energy metabolism during conjugation in Hfr X F^- matings of *Escherichia coli* K-12 (*Bacteriol. Proc.*, 1967, p. 55)	American Society for Microbiology (67th Annual Meeting), New York City
Darden, E. B., Jr.	Effects of radiation on biological organisms	U.S. Army Nuclear Science Seminar (7th Annual), Oak Ridge, Tenn.
Darden, E. B., Jr. (K. W. Christenberry, M. C. Jernigan, J. W. Conklin, R. S. Bender, and A. C. Upton)	Cataractogenesis in mice following exposure to 35–60 Mev protons as compared with 300 kvp X rays [*Radiation Res.* **31**, 661 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
de Serres, F. J.	The effect of various recovery mechanisms on the spectrum of radiation-induced mutations	Brookhaven Symposium on Recovery and Repair Mechanisms in Radiobiology, Upton, N.Y.
	The radiation experiments on the Biosatellite II mission	Symposium on Gravity and the Organism, Sterling Forest Conference Center, Tuxedo, N.Y.
de Serres, F. J. (M. A Bender, F. J. de Serres, P. Carolyn Gooch, Ida R. Miller, and Sohei Kondo)	The Gemini XI S-4 radiation spaceflight interaction experiments [*Radiation Res.* **31**, 638–39 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
de Serres, F. J. (and H. V. Malling)	Relation between type of base-pair transition and complementation pattern in nitrous acid-induced *ad-3B* mutants of *Neurospora crassa* [*Genetics* **56**, 555 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
de Serres, F. J. (H. V. Malling and F. J. de Serres)	Identification of the spectrum of X-ray-induced intragenic alterations at the molecular level in *Neurospora crassa* [*Radiation Res.* **31** (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Doherty, D. G.	Chemical protection against radiation damage: A review	Tenth Anniversary Bone Marrow Conference, Oak Ridge, Tenn.
	The Inhalation Carcinogenesis Program	University of Rochester, Rochester, N.Y.
	Radiation protection	U.S. Army Nuclear Science Seminar (7th Annual), Oak Ridge, Tenn.
Dolin, M. I.	Kinetics of malic-lactic transhydrogenase	VIIth International Congress of Biochemistry, Tokyo, Japan
Donnellan, J. E., Jr.	Effects of ultraviolet radiation on microbial DNA	Gordon Research Conference on Microbiological Deterioration, Tilton, N.H.
Donnellan, J. E., Jr. (R. S. Stafford and J. E. Donnellan, Jr.)	The ultraviolet photochemistry of germinating spores (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 80)	Biophysical Society (11th Annual Meeting), Houston, Tex.
Dumont, J. N.	Electron microscopic study of oocyte development and yolk formation in the horseshoe crab, *Limulus polyphemus*	University of Windsor, Windsor, Ontario, Canada
	Oocyte differentiation in the annelid, *Enchytraeus albidus*	American Society for Cell Biology, Denver, Colo.
Ehling, U. H.	Mammalian genetics	ORAU Summer Institute in Radiation Biology, Oak Ridge, Tenn.
	Radiation biology – genetic effects	ORAU Summer Institute in Health Physics, Oak Ridge, Tenn.
Ehling, U. H. (R. B. Cumming, and H. V. Malling)	Chemically induced dominant lethals in male mice [*Genetics* **56**, 557 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Einstein, J. R.	The combination of isomorphous replacement and anomalous scattering data in phase determination for protein crystals	National Meeting of the American Crystallographic Association, Minneapolis, Minn.
Elrod, L. H. (N. G. Anderson, L. H. Elrod, L. C. Patrick, and J. C. Shinpaugh)	Isolation of nuclei using reoriented gradients	77th Annual Meeting of the Tennessee Academy of Science, Cookeville

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Epler, J. L.	The genetic code in Neurospora	(1) Iowa State University, Ames (2) University of Arkansas, Fayetteville
	Transfer of genetic information: the genetic code	University of Missouri, Columbia
Epler, J. L. (G. David Novelli, and W. E. Barnett)	Coding properties of Neurospora mitochondrial and cytoplasmic leucine transfer RNA's	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Finamore, F. J.	Nucleotide metabolism and brine shrimp embryogenesis	(1) Drake University, Des Moines, Ia. (2) Cornell College, Mount Vernon, Ia. (3) Grinnell College, Grinnell, Ia.
	Nucleotide metabolism and embryogenesis of *Artemia salina*	2nd International Conference on Cell Synchrony, Oak Ridge, Tenn.
	The role of nucleotides in embryonic differentiation	(1) Clemson College, Clemson, S.C. (2) Louisiana State University, Baton Rouge
Finamore, F. J. (J. S. Clegg, A. H. Warner, and F. J. Finamore)	Evidence for the function of P^1, P^4-diguanosine 5′-tetraphosphate in the development of *Artemia salina* [*Federation Proc.* **26**, 604 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Chicago
Fisher, C. R.	Determination of the enzymatic functions controlled by the *ad-3A* and *ad-3B* loci in *Neurospora crassa* [*Genetics* **56**, 560 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Fisher, J. W.	Erythropoietin inhibitor in kidney extracts and plasma from anemic uremic human subjects	American Society of Hematology (10th Annual Meeting), Toronto, Ontario, Canada
Fisher, W. D.	Biophysical separations – The isolation of subcellular particles	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
Fisher, W. D. (H. I. Adler, W. D. Fisher, and Amikam Cohen)	Cell division mutants of *Escherichia coli* (film and discussion)	2nd International Conference on Cell Synchrony, Oak Ridge, Tenn.
Fisher, W. D. (and M. G. Hanna)	Studies on isologous mouse macroglobulin	ORINS Medical Division Seminar, Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Fujimura, R. K. (and Elliot Volkin)	Incorporation of nucleotides into preexisting bacteriophage T5 DNA in the absence of DNA replication	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
	Repair of parental DNA in T5 bacteriophage infected *E. coli* – its possible involvement in recombination of DNA molecules	VIIth International Congress of Biochemistry, Tokyo, Japan
Fuller, R. C.	Bacterial photosynthesis	Michigan State University, East Lansing, Mich.
	Comparative structure and function of photosynthesis	University of Tennessee, Knoxville
	The role of ribose 5-P isomerase in regulation of the Calvin cycle in *Rhodospirillum rubrum*	Conference on Comparative Biochemistry and Biophysics of Photosynthesis, Hakone, Japan
	Use of isotopes in biology and medicine	National Youth Conference on the Atom, Chicago
Fuller, R. C. (N. A. Nugent, and Nicholas Rigopoulos)	Pteridine and photosynthetic electron transport	VIIth International Congress of Biochemistry, Tokyo, Japan
Gassen, H. G. (and Mayo Uziel)	Oligonucleotides obtained by ribonuclease hydrolysis of $tRNA^{Phe}$ (*E. coli* B)	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Gassner, George, III	(1) A paracrystalline body in primary spermatocytes and primary oocyte nurse cells of the scorpion fly, *Panorpa nupitalis* G. (Mecoptera) (read by title) (2) Synaptinemal complexes: Recent findings (read by title)	American Society for Cell Biology, Denver, Colo.
	Synaptinemal complexes in meiotic chromosomes	City of Hope Medical Center, Duarte, Calif.
Goodman, Joan Wright (Gayle C. Bosma, and Sarah G. Shinpock)	Poor growth of parental marrow in old radiation chimeras [*Federation Proc.* **26**, 755 (1967)]	Federation of American Societies for Experimental Biology (Am. Assoc. Immunol.), Chicago
Goodman, Joan Wright (and H. B. Wheeler)	Factors that modify poor growth of parental cells in F_1 hybrid mice	First International Congress of the Transplantation Society, Paris, France
Green, J. G.	Bioengineering – The application of instrumentation to biological problems	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
Grell, E. H.	The deposition of eye pigments at abnormal sites in mutants of *Drosophila melanogaster*	Conference on Genetics, Biochemistry, and Physiology of Insect Eye Color Mutants, Munich, Germany
	Selection of mutants of *Drosophila melanogaster* with altered alcohol dehydrogenase	Genetics Society of America (36th Annual Meeting), Stanford, Calif.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Grell, Rhoda F.	Pairing at the chromosomal level	Symposium on Chromosome Mechanics at the Molecular Level, Gatlinburg, Tenn. (ORNL Biology Division Research Conference)
	Temperature-induced exchange within heterochromatin [*Genetics* **56**, 562 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Gude, W. D.	Autoradiography	ORAU Medical Radioisotopes Course, Oak Ridge, Tenn.
	Basic cell and tissue structure	U.S. Army Nuclear Science Seminar (7th Annual), Oak Ridge, Tenn.
Haber, A. H.	Plant development and senescence	University of South Carolina, Columbia
	Recent applications of ionizing radiation in studies of plant development	University of Massachusetts, Amherst
	Use of ionizing radiations in the analysis of plant growth and development	Dedication of the MSU/AEC Plant Research Laboratory, Symposium on Growth and Development – Form and Function, Michigan State University, East Lansing
Haber, A. H. (and D. E. Foard)	Roles of cell division in plant growth and morphogenesis	Gordon Research Conference on Biochemistry and Agriculture – Control of Growth and Development, Tilton, N.H.
Hager, C. B. (and F. T. Kenney)	Hormonal regulation of enzyme synthesis in isolated, perfused rat livers	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Hamkalo, Barbara A. (and P. A. Swenson)	Effects of ultraviolet irradiation on bacterial respiration and growth	Kentucky-Tennessee Branch of the American Society for Microbiology, Memphis
Hanna, M. G., Jr.	(1) Consequences of the immune response (2) Morphologic changes in spleen lymphatic tissue during an immune reaction	Miami University, Oxford, Ohio
	Morphologic changes in spleen lymphatic tissue during antibody production	(1) Tulane University, New Orleans (2) Louisiana State University, Baton Rouge

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Hard, R. C., Jr.[4] (and R. C. Allen)	Possible use of enzyme markers to follow the genetic stability of donor and host cells in mouse radiation chimeras	Tenth Anniversary Bone Marrow Conference, Oak Ridge, Tenn.
Hard, R. C., Jr.[4] (Birgitta Kullgren, Dorothy J. Moore, and R. C. Allen)	Esterase isoenzyme changes in radiation chimeras [*Federation Proc.* **26**, 755 (1967)]	Federation of American Societies for Experimental Biology (Transplantation Soc.), Chicago
Harris, W. W. (Frances L. Ball, Helen Coates, G. B. Cline, N. G. Anderson, and R. M. Chanock)	Ultrastructure of respiratory syncytial virus (Long strain)	4th Annual Electron Microscopy Symposium of the Louisiana Society for Electron Microscopy, New Orleans
Harrison, A. P., Jr.	Photodynamic effects of benzo(a)pyrene on *E. coli*	(1) University of Missouri, Columbia (2) University of South Carolina, Columbia
	Protein and DNA damage to *Escherichia coli* by 350 Å illumination with benzo(a)pyrene, a carcinogenic hydrocarbon	Brooklyn College, Brooklyn, N.Y.
Harrison, A. P., Jr. (and Vivian E. Raabe)	Leakage of protein components from *Escherichia coli* (*Bacteriol. Proc.*, 1967, p. 132)	American Society for Microbiology (67th Annual Meeting), New York City
Hellman, Alfred (L. J. Wopschall, and J. G. Farrelly)	Aminoacyl sRNA synthetase levels in viral and non-viral infected mammalian cells (*Bacteriol. Proc.*, 1967, p. 141)	*Ibid.*
Hellman, Kiki B. (Alfred Hellman, R. B. Cumming, and G. David Novelli)	Physiological parameters of L-M cells propagated *in vivo* and *in vitro* (*Bacteriol. Proc.*, 1967, p. 171)	*Ibid.*
Hollaender, Alexander	Biological research in the Far East and Latin America	Oak Ridge Section Meeting, Scientific Research Society of America, Oak Ridge, Tenn.
	Biology Division activities	Cancer Institute, Theagenion Memorial, Thessaloniki, Greece
	Introduction	2nd International Conference on Cell Synchrony, Oak Ridge, Tenn.
	Modern biology	Conference on Science for Clergymen, ORAU, Oak Ridge, Tenn.

[4]Medical College of Virginia, Richmond.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Hollaender, Alexander	Opening address	Symposium on the Peaceful Uses of Atomic Radiation, Rio de Janeiro, Brazil
	(1) Opening remarks (2) Closing remarks	International Symposium on Basic Mechanisms in Photochemistry and Photobiology, Caracas, Venezuela
	Studies going on in the Biology Division, Oak Ridge National Laboratory	F. Hoffman-La Roche and Co., Basle, Switzerland
	Work at the Biology Division, Oak Ridge National Laboratory	(1) Siriraj Medical School, Bangkok, Thailand (2) University of Dacca, Dacca, Pakistan (3) The Jute Institute, Dacca, Pakistan (4) A.E.C. Institute, Lahore, Pakistan (5) University of Chandigarh, Chandigarh, India (6) University of Rajasthan, Jaipur, India (7) Bhabha Atomic Research Centre, Bombay, India
Holleman, J. W.	Amino acid sequence of the respiratory protein, hemerythrin	University of Tennessee, Knoxville
Jacobson, Ann B.	Studies on proplastid ribosomes	University of Tennessee, Knoxville
Jacobson, K. Bruce	Isoenzymes of alcohol dehydrogenase in Drosophila	(1) University of Tennessee, Knoxville (2) Osaka Prefectural Institute for Radiation Research, Osaka, Japan
Jacobson, K. Bruce (and Virginia L. Holten)	Interaction of tRNA with synthetases and codons (read by title)	VIIth International Congress of Biochemistry, Tokyo, Japan
Jacobson, K. Bruce (and J. B. Murphy)	The isoenzymic forms of alcohol dehydrogenase from Drosophila: Effect of butanol [*Federation Proc.* **26**, 558 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Chicago
	Isoenzymic forms of alcohol dehydrogenase from *Drosophila melanogaster:* Their isolation and interconversion	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Jenkins, V. K.	The effect of estradiol on hemopoiesis in the RF mouse	Tenth Anniversary Bone Marrow Conference, Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Jones, R. T. (and Roy Curtiss III)	*In vivo* genetic instability of *E. coli* in mice	Kentucky-Tennessee Branch of the American Society for Microbiology, Memphis
Kenney, F. T.	Hormonal regulation of liver enzyme synthesis	(1) Vanderbilt University Medical School, Nashville, Tenn. (2) VIIth International Congress of Biochemistry, Tokyo, Japan (3) Fifth Gunma Symposium on Endocrinology, Kusatsu Spa, Japan (4) Tokushima University Medical School, Tokushima, Japan (5) Protein Research Laboratory, Osaka University, Osaka, Japan
	Regulation of enzyme synthesis and degradation in mammalian tissues	(1) University of Missouri Medical School, Columbia (2) Biology Division, Argonne National Laboratory, Chicago
	Regulation of enzyme synthesis in mammalian liver	(1) Marquette University, Milwaukee (2) University of Minnesota Medical School, Minneapolis
	Requirement for protein synthesis in the turnover of a soluble protein	(1) Gordon Research Conference on Biochemical and Structural Aspects of Self-Degradative Processes in Cells, Andover, N.H. (2) National Cancer Institute, Tokyo, Japan
	Turnover of rat liver tyrosine transaminase *in vivo:* Inhibition by protein synthesis inhibitors [*Federation Proc.* **26**, 347 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Chicago
Khym, J. X. (and Mayo Uziel)	The use of the cetyltrimethylammonium cation in nucleotide sequence analyses of transfer ribonucleic acids	VIIth International Congress of Biochemistry, Tokyo, Japan
	The use of the cetyltrimethylammonium cation in terminal sequence analyses of ribonucleic acids	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Kimball, R. F.	The effect of repair on mutation	Medical Center, University of Colorado, Boulder
	Opening remarks	2nd International Conference on Cell Synchrony, Oak Ridge, Tenn.
Kretchmar, A. L.	Serine metabolism in radiation chimeras [*Exptl. Hematol.* **13**, 33 (1967)] [*Exptl. Hematol.* **14**, 41–42 (1967)]	(1) Bone Marrow Conference, Chicago (2) Conference on Bone Marrow Transplantation and the Physiology of Hematopoietic Tissues, Paris, France
Lanier, W. B.	Radiation-sensitive mutants of *Aspergillus nidulans*	University of Maryland, College Park
Leibo, S. P.	Freezing of biological systems	(1) Oklahoma State University, Stillwater (2) Tuskegee Institute, Tuskegee Institute, Ala.
Leibo, S. P. (and Peter Mazur)	Freezing of bacteriophage T4B (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 135)	(1) Biophysical Society (11th Annual Meeting), Houston, Tex. (2) Society for Cryobiology (4th Annual Meeting), Washington, D.C.
Lindsley, D. L.	The genetic control of recombination in Drosophila	Conference on Replication and Recombination of Genetic Materials, Canberra, Australia
Liverman, J. L.	Bioengineering at ORNL	Knoxville–Oak Ridge Section of the American Institute of Chemical Engineers Seminar, Oak Ridge, Tenn.
	Biomedical programs in Oak Ridge	Reactor Division Seminar, ORNL, Oak Ridge, Tenn.
	Chairman, Dedication Ceremonies, University of Tennessee Memorial Research Center – Symposium on Creativity in Scientific Research	University of Tennessee Memorial Research Center, Knoxville
	Chairman's remarks	Meeting on Centrifugal Techniques for Vaccine Purification, Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Liverman, J. L.	Future developments in biomedicine at Oak Ridge National Laboratory	East Tennessee Chapter of the Health Physics Society, Oak Ridge, Tenn.
	Opening remarks	(1) Viral, Tumor, and Transplantation Antigen Isolation Conference, Oak Ridge, Tenn. (2) Symposium on Urinary Constituents of Low Molecular Weight, ORNL, Oak Ridge, Tenn.
	Pollution	(1) Southern Regional Educational Board seminar on The Nuclear Revolution, Oak Ridge, Tenn. (2) Conference on Science for Clergymen, ORAU, Oak Ridge, Tenn.
Longworth, J. W.	Excited state interactions in macromolecules	International Symposium on Basic Mechanisms in Photochemistry and Photobiology, Caracas, Venezuela
	Luminescence of polypeptides and proteins	Symposium on Low Temperature Luminescence of Biologic Macromolecules, National Institutes of Health, Bethesda, Md.
Makinodan, Takashi	Aging and immunological competence	University of Buenos Aires Institute of Medical Research, Buenos Aires, Argentina
	Cytokinetics of immune response	Medical Faculty, University of Chile, Santiago
	Genetic aspects of immune response	Chilean Society of Genetics, Santiago
	Growth and senescence of immune competence	University of Pittsburgh, Pittsburgh, Pa.
	Immune competence and aging	Northwestern University Medical School, Evanston, Ill.
	Radiation immunology	Radiation Physiology Course, University of Tennessee Institute of Radiation Biology, Knoxville

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Malling, H. V.	Chemical mutagens as a genetic hazard in the human population	American Industrial Hygiene Association, Tennessee Valley Section, Crossville
Malling, H. V. (and F. J. de Serres)	*N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) as a mutagenic agent for *Neurospora crassa* [*Genetics* **56**, 575 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Mattingly, Ellen	Chromosome cytology	University of Tennessee, Knoxville
	Polytene chromosomes	Quincy College, Quincy, Ill.
Mattingly, Ellen (and Curtis Parker)	Relation of chromosome puffs to larval cycle in *Rhynchosciara* sp	American Society for Cell Biology, Denver, Colo.
Mazur, Peter	Effects of freezing on microorganisms and bacteriophage	Exobiology Section, Ames Research Center, Moffett Field, Calif.
	Physical and chemical events underlying injury in frozen-thawed cells	Symposium on Cryosurgery, Los Angeles
	Physical and chemical factors underlying cellular injury at subzero temperatures	Conference on Cryobiology and Cyrosurgery, State University of New York, Buffalo
Mazur, Peter (and R. H. Miller)	Survival of supercooled yeast	4th Annual Meeting of the Society for Cryobiology, Washington, D.C.
Mazur, Peter (and Janice J. Schmidt)	Effect of cooling and warming velocities on the survival of frozen and thawed cells of *Escherichia coli*	*Ibid.*
McDevitt, D. S.	The lens-specific proteins and amphibian lens development	(1) School of Veterinary Medicine, University of Pennsylvania, Philadelphia (2) Rensselaer Polytechnic Institute, Troy, N.Y. (3) Middlebury College, Middlebury, Vt.
McDevitt, D. S. (Isaura Meza, and Tuneo Yamada)	An immunofluorescence study of the localization of γ crystallins during embryonic lens development	American Society for Cell Biology, Denver, Colo.
McGrath, R. A.	Cellular repair of irradiated DNA	University of Missouri Medical Center, Columbia
	DNA repair	Donner Laboratory Seminar, University of California, Berkeley

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
McGrath, R. A. (and R. W. Williams)	Interruptions in single-stranded DNA (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 40)	Biophysical Society (11th Annual Meeting), Houston, Tex.
Merriam, J. R.	The initiation of nonhomologous chromosome pairing before exchange in female *Drosophila melanogaster* [*Genetics* **56**, 577 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Miller, O. L., Jr.	DNA and nucleolar structure in amphibian oocytes	Carnegie Institution of Washington, Baltimore
	Nucleolar composition and structure	University of Pennsylvania, Pittsburgh
	The nucleolus – its structure and function	Southeastern Electron Microscopy Society (Spring Meeting), Atlanta, Ga.
	Observations on isolated components of amphibian oocyte nucleoli	American Society for Cell Biology, Denver, Colo.
	Structure and function of the amphibian oocyte nucleolus	(1) Duke University, Durham, N.C. (2) Rockefeller University, New York City (3) State University of New York, Stony Brook
Modak, S. P.	Autoradiographic and microspectrophotometric analyses of lens cell differentiation	Putnam Memorial Hospital Institute for Medical Research, Bennington, Vt.
	Cell replication and cell death during lens development	University of the Pacific, Stockton, Calif.
	DNA synthesis and DNA loss during lens differentiation	Donner Laboratory, University of California, Berkeley
	DNA synthesis and mitotic activity during differentiation and development of chick lens	American Society for Cell Biology, Denver, Colo.
Modak, S. P. (R. C. von Borstel, S. P. Modak, and F. J. Bollum)	Cytochemical localization of sites of RNA synthesis using RNA polymerase	*Ibid.*
Montour, J. L.	Abscopal radiation damage to the chick thymus and bursa of Fabricius	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Nelson, R. L.	Animal care equipment	American Association for Laboratory Animal Science (18th Annual Meeting), Washington, D.C.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Nöthiger, Rolf	Problems of determination and differentiation in imaginal discs of Drosophila	(1) University of Chicago, Chicago (2) Johns Hopkins University, Baltimore
Novelli, G. David	Changes in rat liver tRNA during ethionine carcinogenesis	National Cancer Institute, Kanazawa, Japan
	Discussant	American Cancer Society Symposium on Developmental Biology of Neoplasia, Philadelphia
	Large and small scale studies on tRNA	Indiana University, Bloomington
	Macromolecular separations	(1) National Institute of General Medical Sciences, Bethesda, Md. (2) University of Georgia, Athens
	Separation of transfer RNA	National Cancer Institute, Tokyo, Japan
	Some recent studies with transfer RNA	(1) Massachusetts Institute of Technology, Cambridge (2) Rockefeller University, New York (3) Institute for Muscle Diseases, New York City
Novelli, G. David (A. D. Kelmers, J. F. Weiss, C. Hancher, H. Weeren, and E. F. Phares)	The preparation of individual transfer RNA's in gram quantities	VIIth International Congress of Biochemistry, Tokyo, Japan
Nunley, C. E.	Biological use of the ultracentrifuge	Knoxville–Oak Ridge Section of the American Institute of Chemical Engineers Seminar, Oak Ridge, Tenn.
Nunley, C. E. (and G. B. Cline)	Gas sterilization of contained virus isolation system	Sixth Annual Technical Meeting of the American Association for Contamination Control, Washington, D.C.
Oakberg, E. F.	Mammalian gametogenesis and species comparisons in radiation response of the gonads	IAEA Study Group Meeting on the Effects of Ionizing Radiations on Meiotic Systems, Vienna, Austria

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Oakberg, E. F.	Radiation damage to the gonads	Radiation Physiology Course, University of Tennessee, Knoxville
	Radiation effects on human testes	ORINS Medical Division Seminar, Oak Ridge, Tenn.
	(1) Radiation genetics (2) Radiation response of gonads	Graduate Course in Radiation Biology, University of California, Berkeley
	Relative biological effectiveness of gamma rays, X rays, protons, and neutrons for spermatogonial killing	First International Symposium on Biological Interpretation of Dose from Accelerator-Produced Radiation, Lawrence Radiation Laboratory, University of California, Berkeley
	RNA synthesis in mouse oocytes [*Genetics* **56**, 580 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Oakberg, E. F. (presented paper by W. L. Russell)	Recent studies on the genetic effects of radiation in mice	First International Symposium on Biological Interpretation of Dose from Accelerator-Produced Radiation, Lawrence Radiation Laboratory, University of California, Berkeley
Odell, T. T., Jr.	Maturation of megakaryocytes	(1) University of Tennessee, Knoxville (2) UT-AEC Agricultural Research Laboratory, Oak Ridge, Tenn.
Odell, T. T., Jr. (and C. W. Jackson)	Megakaryocyte maturation	Third Conference on Blood Platelets, Oak Ridge, Tenn.
	Polyploidy and maturation of megakaryocytes [*Federation Proc.* **26**, 745 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Exptl. Pathol.), Chicago
Odell, T. T., Jr. (C. W. Jackson, and Rebecca S. Reiter)	Generation cycle of rat megakaryocytes (read by title)	American Society of Hematology (10th Annual Meeting), Toronto, Ontario, Canada
Ortwerth, B. J. (and G. David Novelli)	Ethylation of rat liver tRNA by ethionine	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Palmer, Winifred G.	Lens cell differentiation and protein synthesis	Wilmer Ophthalmological Institute, Baltimore, Md.
Palmer, Winifred G. (and John Papaconstantinou)	Isolation and characterization of α-crystallin subunits (Abstr. of papers, 154th Meet., Am. Chem. Soc., 1967, p. 94C)	American Chemical Society (154th National Meeting), Chicago
Papaconstantinou, John	The biochemistry of lens cell differentiation	University of Connecticut, Storrs
	Immunochemical and biochemical analysis of the regulation of protein synthesis during lens cell differentiation	Symposium on Differentiation and Immunology, International Society for Cell Biology, Gatlinburg, Tenn.
	Molecular aspects of lens cell differentiation	(1) Institute for Developmental Biology, University of Colorado, Boulder (2) Institute for Cancer Research, Philadelphia
	Regulation of protein synthesis in lens cell differentiation	Symposium on Lens Proteins and Related Subjects, University of Ghent, Ghent, Belgium
	The stabilization of mRNA templates during growth and differentiation of the lens	University of Illinois, Urbana
Papaconstantinou, John (and J. W. Carr)	Crystallin synthesis by lens epithelial cells in tissue culture	American Association for the Advancement of Science (Am. Soc. Zool.), New York City
Parker, D. R.	Induced meiotic exchange – some observations on its nature and consequences	University of Wisconsin, Madison
Pfuderer, Peter	Configuration studies on polyribosomes	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
	Electron microscopy of isolated polysomes	(1) University of Alabama Medical Center, Birmingham (2) Auburn University, Auburn, Ala. (3) Vanderbilt University, Nashville, Tenn.
	The incorporating activity of isolated polysomes	University of Alabama, Birmingham

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Popp, R. A.	(1) Chemical basis for hemoglobin variants in mice (2) Esterase variants in mice with emphasis on their role in development	Marquette University, Milwaukee
	Genetic and molecular bases for hemoglobin variants in mice	University of Virginia, Charlottesville
	Isolation and characterization of transplantation antigens	Oak Ridge Associated Universities, Oak Ridge, Tenn.
	Isolation of transplantation antigens from mouse spleen	Viral, Tumor, and Transplantation Antigen Isolation Conference, Oak Ridge, Tenn.
Puccia, Egidio	Some aspects of amphibian yolk platelets ultrastructure	Ninth Southeastern Developmental Biology Conference, New Orleans
Rahn, R. O.	Photochemistry of DNA	Solid State Physics Group, ORNL, Oak Ridge, Tenn.
Rahn, R. O. (and J. L. Hosszu)	Influence of temperature on thymine dimer formation in DNA and poly dT	International Symposium on Basic Mechanisms in Photochemistry and Photobiology, Caracas, Venezuela
	Pyrimidine dimer formation at 77K in polynucleotides (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 37)	Biophysical Society (11th Annual Meeting), Houston, Tex.
Rahn, R. O. (and J. W. Longworth)	Low-temperature luminescence of polynucleotides	Symposium on Recent Developments in Research Methods and Instrumentation, National Institutes of Health, Bethesda, Md.
Randolph, M. L.	Elemental composition of biological tissues as a limiting factor in specification of dose [*Radiation Res.* **31**, 621 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Reel, J. R.	The effect of actinomycin D on tyrosine transaminase synthesis in hydrocortisone-induced and non-induced hepatoma cell cultures	Division of Pharmacology, Eli Lilly & Co., Indianapolis, Ind.
	Regulation of tyrosine transaminase synthesis by hydrocortisone in cultured Reuber H-35 hepatoma	Duke University Medical Center, Durham, N.C.
Reel, J. R. (and F. T. Kenney)	Hormonal control of enzyme synthesis in hepatoma cell cultures	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Regan, J. D.	Aspects of the effects of UV irradiation on human cells	University of Tennessee Medical School, Memphis
	Evidence for photoreactivation in marine fish cells *in vitro*	Annual Meeting of the Tissue Culture Association, Symposium on Defense Mechanisms in Lower Organisms, Philadelphia
	Repair of ultraviolet-induced lesions in cells *in vitro*	(1) Greensboro College, Greensboro, N.C. (2) University of North Carolina, Chapel Hill (3) Institute of Therapeutic Biochemistry, Frankfort, Germany (4) Institute of Radiation Biology, Karlsruhe, Germany
Regan, J. D. (and J. E. Trosko)	The fate of ultraviolet light-induced thymine-containing dimers in human cells grown *in vitro* [*Radiation Res.* **31**, 548 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Reimer, C. B. (R. S. Baker, R. M. VanFrank, and T. E. Newlin)	Purification of large quantities of influenza virus by density gradient centrifugation	Meeting on Centrifugal Techniques for Vaccine Purification, Oak Ridge, Tenn.
Richter, C. B.	A facility design for pathogen-free, inbred mouse production	American Association for Contamination Control, 6th Annual Technical Meeting and Exhibit, Washington, D.C.
Richter, C. B. (M. L. Simmons, C. B. Richter, R. W. Tennant, and J. A. Franklin)	Controlling infectious diseases in a laboratory mouse breeding colony [Abstr. of papers, 18th Annual Meet., AALAS, No. 60 (1967)]	American Association for Laboratory Animal Science (18th Annual Meeting), Washington, D.C.
Riggsby, W. S. (and R. K. Fujimura)	Fractionation of DNA on nitrocellulose columns	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Rogers, Stanfield	Concerning the implications of passenger viruses for genetics	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
Rogers, Stanfield (and J. H. Evans)	A mutant of the Shope papilloma virus and its influence on arginase activity [*Proc. Am. Assoc. Cancer Res.* **8**, 57 (1967)]	Am. Assoc. Cancer Res. (58th Annual Meeting), Chicago
Rosen, Lawrence (B. J. Ortwerth, and G. David Novelli)	Characterization of labeled rat-liver RNA following the injection of ^{14}C-L-ethionine	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Russell, Liane B.	Complementation mapping of a small region of linkage group 2 of the mouse [*Genetics* **56**, 585 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
	Re-evaluation of the Lyon hypothesis on the basis of evidence from classical genetic markers	Fourth Basel Colloquium on Mammalian Sex Chromosomes in Differentiation and Development, Basel, Switzerland
	Sex chromosomes of mammals	Southern Regional Educational Board seminar on The Nuclear Revolution, Oak Ridge, Tenn.
	The use of sex-chromosome aberrations for measuring radiation effects in mammals	IAEA Study Group Meeting on The Effects of Ionizing Radiations on Meiotic Systems, Vienna, Austria
Russell, W. L.	Effects of ionizing radiation on mammalian genetics	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tenn. (2 lectures)
	Genetic effects from radiation exposure	Lecture to Health Physics Students, ORAU Special Training Building, Oak Ridge, Tenn.
	Genetic expression of radiation injury	Brookhaven Symposium on Recovery and Repair Mechanisms in Radiobiology, Upton, N.Y.
	Mutagenesis and radiation	Buffalo School of Medicine, State University of New York, Buffalo
	Radiation and mutation rates	Symposium on the Peaceful Uses of Atomic Radiation, Rio de Janeiro, Brazil
	Recent advances in mammalian radiation genetics	Roswell Park Memorial Institute, Buffalo, N.Y.
	Recent findings in mammalian radiation genetics	Health Physics Society (12th Annual Meeting), Washington, D.C.
	Recent genetics research and relation of results to hazard evaluation	East Tennessee Chapter of the Health Physics Society, Oak Ridge, Tenn.
	Recent studies on the genetic effects of radiation	Texas Woman's University, Denton
Russell, W. L. (and Liane B. Russell)	Genetics	Conference on Science for Clergymen, ORAU, Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Serrano, L. J.	Semiautomatic recording and computer processing of mouse breeding data	American Association for Laboratory Animal Science (18th Annual Meeting), Seminar on Data Processing in Production and Research Animal Colonies, Washington, D.C.
Setlow, Jane K.	The molecular basis of ultraviolet inactivation and photoreactivation	Smithsonian Institute Lecture, Washington, D.C.
	Photochemistry of DNA	Radiation Biology Institute, ORAU, Oak Ridge, Tenn.
Setlow, Jane K. (and F. J. Bollum)	The minimum size of the substrate for the photoreactivating enzyme (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 85)	Biophysical Society (11th Annual Meeting), Houston, Tex.
Setlow, R. B.	(1) Effects of ultraviolet radiation on viruses and cells (2) Evidence that pyrimidine dimers affect biological systems (3) The importance of DNA in radiobiology (4) Modern trends in biology (5) The photochemistry of DNA (6) The reactions of enzymes on irradiated DNA (7) The repair of DNA	Atomic Energy Center, Lahore, Pakistan
	The excision of pyrimidine dimers *in vivo* and *in vitro*	International Conference on Replication and Recombination of Genetic Material, Canberra, Australia
	Modern trends in biology	University of Punjab, Lahore, Pakistan
	Molecular repair systems	Columbia University, New York City
	Photochemistry and photobiology of polynucleotides	(1) Gordon Research Conference on Nucleic Acids, New Hampton, N.H. (2) Westchester Chemical Society, Yonkers, N.Y.
	Photochemistry of polynucleotides	University of Melbourne, Melbourne, Australia
	Photoproducts in DNA irradiated *in vivo*	International Symposium on Basic Mechanisms in Photochemistry and Photobiology, Caracas, Venezuela
	Repair of DNA	Hebrew University, Jerusalem, Israel

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Setlow, R. B.	Steps in the repair of DNA: Fact and fancy	Brookhaven Symposium on Recovery and Repair Mechanisms in Radiobiology, Upton, N.Y.
Setlow, R. B. (W. L. Carrier, and R. W. Williams)	Strand breaks during excision of pyrimidine dimers *in vivo* (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 83)	Biophysical Society (11th Annual Meeting), Houston, Tex.
Shepard, C. C. (and C. C Congdon)	Increased growth of *Mycobacterium leprae* in the foot pads of thymectomized-irradiated mice	Tenth Anniversary Bone Marrow Conference, Oak Ridge, Tenn.
Shore, R. E.	Distribution of crystallins during lens growth and differentiation	(1) University of Toledo, Toledo, Ohio (2) State University of New York, Buffalo
Shugart, L. R. (Barbara Chastain, and M. P. Stulberg)	Properties of undermethylated transfer RNA from an RC^{rel} mutant of *Escherichia coli*	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Shugart, L. R. (and M. P. Stulberg)	Isolation of phenylalanine tRNA from an RC^{rel} mutant of *Escherichia coli* (*Bacteriol. Proc.*, 1967, p. 116)	American Society for Microbiology (67th Annual Meeting), New York City
Simmons, M. L.	The management of the laboratory mouse	Marquette University, Milwaukee
	Proper care and housing of laboratory animals	Miami University, Oxford, Ohio
Simmons, M. L. (C. B. Richter, and R. W. Tennant)	Specific pathogen-free rat production in plastic germfree isolator rooms	International Symposium on Germfree Life in Research, Nagoya, Japan
Smith, L. H. (and T. W. McKinley, Jr.)	Effect of radiation temperature on erythropoietic capacity of mouse bone marrow cells [*Radiation Res.* **31**, 598 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Smith, R. H.	Dominant and recessive lethal mutations induced by mitomycin C in Habrobracon oocytes and sperm [*Genetics* **56**, 591 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Sproston, Thomas	UV radiation effects on fungi	University of Tennessee, Knoxville
Stewart, J. A.	mRNA stabilization in lens cell differentiation	University of California, Los Angeles
Stewart, J. A. (and John Papaconstantinou)	Stabilization of mRNA in lens epithelial cells [*Federation Proc.* **26**, 604 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Chicago
Stine, G. J.	Enzyme studies during the development of the asexual cycle of *Neurospora crassa*	University of California, Los Angeles

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Stine, G. J.	Obtaining synchrony in the production of conidiophores of *Neurospora crassa*	2nd International Conference on Cell Synchrony, Oak Ridge, Tenn.
	Phasing of development and measurement of specific enzymes during the asexual cycle of *Neurospora crassa*	University of Tennessee, Knoxville
	Specific enzyme activities during germination, development, and differentiation in the asexual cycle of *Neurospora crassa*	Rutgers, The State University, New Brunswick, N.J.
Stulberg, M. P.	Function and structure of $tRNA^{Phe}$	(1) Molecular Biology Division, Institute of Cancer Research, Kanazawa National University, Kanazawa, Japan (2) Virology Division, National Cancer Research Institute, Tokyo, Japan
	Interactions of phenylalanyl-RNA synthetase and tRNA	Dedicatory Symposium for the Gortner Laboratory of Biochemistry, University of Minnesota, St. Paul
	Mechanism of phenylalanyl-tRNA formation	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Swenson, P. A.	Delayed response in the photoreactivation of tryptophanase inducibility in *E. coli* B (Abstr. of papers, 11th Annual Meet., Biophys. Soc., 1967, p. 82)	Biophysical Society (11th Annual Meeting), Houston, Tex.
	The effects of ultraviolet radiation on induced enzyme synthesis and metabolism in *E. coli*	Ames Research Laboratory, Moffett Field, Calif.
	Ultraviolet photobiology	University of Tennessee Radiation Biology Course, Knoxville
Szakal, A. K. (and M. G. Hanna, Jr.)	The ultrastructure of antigen localization and virus-like particles in mouse spleen germinal centers	Tenth Anniversary Bone Marrow Conference, Oak Ridge, Tenn.
ten Bosch, J. J.	X-ray induced radical production in rat-tail tendon collagen and gelatins	National Institutes of Health, Bethesda, Md.
Thomas, D. B. (and C. C Congdon)	The source of thymic precursor-cells [*J. Anat.* **101**, 614 (1967)]	Anatomical Society of Great Britain and Ireland (Annual General Meeting), London, England
	Spontaneous recovery in the thymus after supralethal whole-body X-irradiation [*J. Anat.* **101**, 614 (1967)]	*Ibid.*

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Tyndall, R. L.	Some ramifications of leukemia virus infection	North Carolina State University, Raleigh
Tyndall, R. L. (and R. C. Allen)	Altered esterase zymograms associated with leukemia virus infection	Tissue Culture Association (Annual Meeting), Philadelphia
Tyndall, R. L. (J. A. Otten, Ernestine Teeter, and N. D. Bowles)	Inhibition of solid tumor formation by immunization with leukemic spleen extracts (*Bacteriol. Proc.*, 1967, p. 153)	American Society for Microbiology (67th Annual Meeting), New York City
Upton, A. C.	Biological effects of low levels of radiation	ORAU Medical Radioisotope Course, Oak Ridge, Tenn.
	Current highlights of cancer research	American Cancer Society (Anderson County Unit), Clinton, Tenn.
	Effects of radiation on humans	University of Tennessee, Knoxville
	Highlights in cancer research	East Tennessee Section, American Cancer Society, Knoxville
	Pathology of radiation injury	University of Chicago Medical School, Chicago
	Radiation carcinogenesis	12th Annual Meeting of the Health Physics Society, Washington, D.C.
	Radiation and carcinogenesis	Symposium on the Peaceful Uses of Atomic Radiation, Rio de Janeiro, Brazil
	The role of radiation in the etiology of leukemia	International Conference on Leukemia-Lymphoma, Ann Arbor, Mich.
	Studies on cancer research	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
	Mechanisms of radiation carcinogenesis	Peruvian Society of Oncology, Lima, Peru
	Carcinogenic effects of ionizing radiation	University of Ribeirão Prêto, Ribeirão Prêto, Brazil
Upton, A. C. (and J. W. Conklin)	Coleukemogenic effects of triethylene melamine and X rays on the mouse thymus [*Proc. Am. Assoc. Cancer Res.* **8**, 69 (1967)]	American Association for Cancer Research, Chicago
	Preliminary observations on the life-shortening effects of triethylene melamine and X rays administered in combination to mice	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Uziel, Mayo	Methods in the study of the structure of nucleic acids	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Uziel, Mayo	Structure of transfer RNA	University of British Columbia, Vancouver, Canada
Uziel, Mayo (and H. G. Gassen)	Oligonucleotide sequences in $tRNA^{Phe}$ (*E. coli* B)	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Vann, D. C.	Antibody synthesis after exposure of lymphoid cells to 10,000 R [*Federation Proc.* **26**, 751 (1967)]	Federation of American Societies for Experimental Biology (Am. Assoc. Immunol.), Chicago
Vodopick, Helen[5] (C. C Congdon, C. L. Edwards, Nazareth Gengozian, B. Nelson, and G. A. Andrews)	Graft-versus-host reaction in man produced by thoracic-duct lymphocytes [*Exptl. Hematol.* **13**, 38 (1967)]	Bone Marrow Conference, Chicago
Volkin, Elliot	Biochemical evidence of mechanism of genetic recombination	University of Georgia, Athens
von Borstel, R. C.	Chromosome breakage and cell killing by radiation	(1) Pakistani Atomic Energy Center, Karachi (2) Pakistani Atomic Energy Center, Lahore (3) Pakistani Atomic Energy Center, Pinstech (4) Siriraj Medical School, Bangkok, Thailand
	Chromosome breakage and dominant lethality	Sirivai Hospital, Bangkok, Thailand
	Control of adult pests by the irradiation-of-male method	Symposium on the Use of Isotopes and Radiation in Entomology, Vienna, Austria
	Developmental genetics — gene control during growth and differentiation	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
	Dicentric translocations as a component of dominant lethality in irradiated Habrobracon sperm	International Chromosome Conference, Oxford, England
	Dominant lethality	Division of Biology and Medicine, U.S. Atomic Energy Commission, Washington, D.C.

[5]Medical Division, ORINS, ORAU.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
von Borstel, R. C.	The irradiation-of-male method of insect control	(1) Pakistani Atomic Energy Center, Tandojam (2) University of Lahore, Lahore, Pakistan (3) Thailand Office of Atomic Energy for Peace, Bangkok (4) National Biological Institute, Bogor, Indonesia (5) Bhabha Atomic Research Center, Bombay, India
	Nature of spontaneous mutations in radiation-sensitive strains of yeast	Pakistani Atomic Energy Center, Lahore
	Relation of super-suppressor genes to transfer RNA in yeast	University of Delaware, Newark
	The relation of super suppressors to transfer-RNA synthesis	(1) Pakistani Atomic Energy Center, Lahore (2) University of Dacca, Dacca, Pakistan (3) Medical College of the University of Indonesia, Djakarta (4) National Biological Institute, Bogor, Indonesia (5) Institute of Technology, Bandung, Indonesia (6) Bhabha Atomic Research Center, Bombay, India (7) The Pusa Institute, New Delhi, India
	Space biology	(1) Pakistani Atomic Energy Center, Dacca (2) National Biological Institute, Bogor, Indonesia (3) Bhabha Atomic Research Center, Bombay, India (4) University of New Delhi, New Delhi, India
	Super suppressors	(1) University of Pittsburgh, Pittsburgh, Pa. (2) Indian Institute of Science, Bangalore, India

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
von Borstel, R. C. (R. H. Smith, and Anna R. Whiting)	Genetic effects of chronic γ-radiation on Habrobracon sperm [*Radiation Res.* **31**, 615 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Walburg, H. E., Jr. (G. E. Cosgrove, and A. C. Upton)	Influence of the microbial environment on the development of myeloid leukemia in X-irradiated RFM mice [*Radiation Res.* **31**, 552 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Wald, Niel (Sylvia Pan, A. C. Upton, and R. C. Brown)	Evolution of chromosome aberrations in radiation-induced and passaged mouse granulocytic leukemia [*Radiation Res.* **31**, 600 (1967)]	Radiation Research Society (15th Annual Meeting), San Juan, Puerto Rico
Wallace, R. A.	A serum lipophosphoprotein produced by vitellogenic females and estrogenized males of *Xenopus laevis*	American Society for Cell Biology, Denver, Colo.
	Vitellogenesis	University of Michigan, Ann Arbor
Waters, L. C. (and G. David Novelli)	Studies of leucyl-tRNA during phage infection of *E. coli*	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Waters, L. C. (Elliot Volkin, and G. David Novelli)	Changes in leucyl-tRNA during phage T2 infection [*Federation Proc.* **26**, 733 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Chicago
Webber, B. B. (and H. V. Malling)	Relation between complementation pattern and genetic alteration in *hist-3* mutants of *Neurospora* [*Genetics* **56**, 595 (1967)]	Genetics Society of America (36th Annual Meeting), Stanford, Calif.
Weiss, J. F. (A. D. Kelmers, and M. P. Stulberg)	A new chromatographic system for increased resolution of transfer RNAs [*Federation Proc.* **26**, 734 (1967)]	Federation of American Societies for Experimental Biology (Am. Soc. Biol. Chem.), Chicago
Whiting, P. W.	A diapause-resistant stock of Mormoniella [*ASB Bull.* **14**, 42 (1967)]	Association of Southeastern Biologists, Columbia, S.C.
Whitson, G. L.	Problems in cell division and morphogenesis	UT-AEC-ORAU Oak Ridge Resident Graduate Program, Oak Ridge, Tenn.
	The relation of thymine dimers, dark repair, and photoreactivation to survival in *Tetrahymena pyriformis*	(1) Bryn Mawr College, Bryn Mawr, Pa. (2) University of Tennessee, Knoxville
Whitson, G. L. (A. A. Francis, and W. L. Carrier)	Production of thymine dimers and repair in ultraviolet-irradiated *Tetrahymena pyriformis*, strain GL-C	Society of Protozoologists, Toronto, Canada
Whitson, G. L. (A. A. Francis, W. L. Carrier, and K. L. Grandi)	Survival in the absence of *in vivo* photoreactivation of thymine dimers in *Tetrahymena pyriformis*	American Society for Cell Biology, Denver, Colo.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Whitson, G. L. (J. G. Green, A. A. Franci, and D. D. Willis)	Fractionation of alcohol-soluble carbohydrates from synchronized *Tetrahymena*	2nd International Conference on Cell Synchrony, Oak Ridge, Tenn.
Wicks, W. D.	Control of enzyme activity during development	(1) Louisiana State University Medical School, New Orleans (2) Louisiana State University, Baton Rouge (3) Texas A&M College, College Station (4) Oklahoma State University, Stillwater (5) St. Louis University, St. Louis (6) University of Kentucky, Lexington (7) Vanderbilt University, Nashville, Tenn. (8) Bowman-Gray School of Medicine, Winston-Salem, N.C.
	Control of enzyme synthesis during development	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
	Hormones: Biochemical regulators	(1) Southern University, Baton Rouge, La. (2) Sweet Briar College, Sweet Briar, Va.
	Induction of tyrosine transaminase in fetal rat liver	University of Alabama, Birmingham
Wittliff, J. L.	Purification and properties of extracellular thiaminase I from *Bacillus thiaminolyticus*	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Wust, C. J.	Cell biology	American Institute of Chemical Engineers, Oak Ridge, Tenn.
Wust, C. J.	Some aspects of antibody biosynthesis	Siena Heights College, Adrian, Mich.
Wust, C. J. (and G. David Novelli)	Binding of ^{125}I-labeled anti-L chain to polysomes of rat spleen *in vitro*	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.
Wust, C. J. (and D. G. Ridner)	Studies on ribosomes in spleens of immunized rats treated with actinomycin D (*Bacteriol. Proc.*, 1967, p. 66)	American Society for Microbiology (67th Annual Meeting), New York City
Yamada, Tuneo	Cellular and molecular aspects of tissue transformation	University of Massachusetts, Amherst

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Yamada, Tuneo	Cellular and molecular events in Wolffian lens regeneration	National Institute of Child Health and Human Development, Bethesda, Md.
	(1) Cellular replication in dedifferentiation and differentiation: A study of lens regeneration (2) Localization of labeled actinomycin D in differentiating lens regenerate cells	VIIIth International Embryological Conference, Interlaken, Switzerland
	Cellular synthetic activities in induction of tissue transformation	CIBA Symposium on Cell Differentiation, London, England
	Control of cellular differentiation	University of Toronto, Toronto, Ontario, Canada
	Sequential changes in cellular synthetic activities associated with tissue transformation	Institute of Physiochemical Biology, Paris, France
	Specific proteins in cytodifferentiation	University of Pennsylvania Medical School, Philadelphia
Yang, Wen-Kuang	Multiple isoaccepting tRNA's in mouse plasma cell tumors	Tuskegee Institute, Tuskegee, Ala.
Yang, Wen-Kuang (and G. David Novelli)	Studies on the multiple isoaccepting transfer-ribonucleic acids in mouse plasma cell tumors	Symposium on Nucleic Acids in Immunology, New Brunswick, N.J.
	Studies on transfer RNA's of a mouse plasma cell tumor	Southeastern Regional Meeting, American Chemical Society, Atlanta, Ga.

VISITING LECTURERS

During the period February 1 through December 31, 1967, 81 seminars were given at the Biology Division by visiting scientists. Fifty of the speakers came from other research organizations in the United States, one from the Chemistry Division of the Oak Ridge National Laboratory, and the remaining thirty from seventeen foreign countries. Countries represented (and the number of speakers from each) were England (10), Chile, Germany, Japan, and Sweden (2 each), and Australia, Belgium, Brazil, Canada, Czechoslovakia, Greece, Italy, The Netherlands, New Zealand, Scotland, Switzerland, and Venezuela (1 each).

In addition to the 81 guest speakers, listed below, there were numerous informal seminars at which Division members spoke about their own research.

SPEAKER	AFFILIATION	SUBJECT
Roy E. Albert	Institute of Environmental Medicine, New York University Medical Center, New York, New York	Radiation skin carcinogenesis in the rat

SPEAKER	AFFILIATION	SUBJECT
Peter Alexander	Chester Beatty Research Institute, London, England	Application of the McGrath and Williams method for the study of repair following X-irradiation of mammalian cells and *Micrococcus radiodurans*
Siegmund Baum	Experimental Pathology Department, Defense Atomic Support Agency, Armed Forces Radiobiology Research Institute, Bethesda, Maryland	Recovery and residual injury in the irradiated polycythemic rat
Antonio Garcia Bellido	Division of Biology, California Institute of Technology, Pasadena, California	Cellular determination and cell affinity in imaginal discs of Drosophila
Irwin Berman	School of Medicine, Department of Anatomy, University of Miami, Miami, Florida	The effects of cytotoxic agents on stem cells
T. J. Bojarski	New England Nuclear Corporation, Boston, Massachusetts	Determination of purity of labelled nucleotides
William Boyle	Division of Immunology, Duke University Medical Center, Durham, North Carolina	Isolation and characterization of cell membrane
B. A. Bridges	Medical Research Council, Radiobiological Research Unit, Harwell, Didcot, Berkshire, England	Induction and repair of UV damage leading to death, true reversion and suppressor mutation in bacteria irradiation in the frozen and unfrozen states
D. M. Brown	University Chemical Laboratory, Cambridge, England	The chemical basis of hydroxylamine mutagenesis
P. R. J. Burch	Environmental Radiation Research Unit, Department of Medical Physics, The University of Leeds, The General Infirmary, Leeds, England	A unified theory of growth and disease
Alice Burton	Department of Biology, Brookhaven National Laboratory, Upton, L.I., New York	Some aspects of the intracellular development of tiny bacteriophages
Ivan L. Cameron	Department of Anatomy, State University of New York, Upstate Medical Center, Syracuse, New York	Cell proliferation and renewal in the tissues of the adult mouse
Teh-hsing Chiu	Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania	L-Rhamnulose 1-phosphate aldolase
Gabriel Chuchani	Scientific Research Institute, Caracas, Venezuela	Effect of amino groups in electrophilic aromatic substitution
B. F. C. Clark	Laboratory of Molecular Biology, Cambridge, England	Initiation of polypeptide synthesis

SPEAKER	AFFILIATION	SUBJECT
C. Vernon Cole	Agricultural Research Service, United States Department of Agriculture, Fort Collins, Colorado	Early products of phosphate uptake in plants
Richard G. Considine	Lobund Laboratories, University of Notre Dame, Notre Dame, Indiana	*In vivo* interferon production by vaccinia virus
S. B. Curtis	Lawrence Radiation Laboratory, University of California, Berkeley, California	Pion radiobiology
Katsuma Dan	Tokyo Metropolitan University, Meguro-ku, Tokyo, Japan	Cytoplasmic factors involved in cell division
J. Desmond Davies	Department of Pathology, University of Cambridge, Cambridge, England	Studies on freezing and freeze-drying of living cells
Daniel Der Vartanian	Institute of Enzyme Research, University of Wisconsin, Madison, Wisconsin	EPR studies on non-heme iron proteins
Raymond Devoret	Department of Radiology, Yale University School of Medicine, New Haven, Connecticut	Indirect ultraviolet induction of prophage
David A. T. Dick	Department of Physiology and Pharmacology, Duke University, Durham, North Carolina	Some studies of ion and water fluxes in a single cell system
Jacques E. Dumont	Laboratory of Nuclear Medicine, School of Medicine, University of Brussels, Brussels, Belgium	Studies on the mechanism of action of thyrotropin
Lawrence Erway	Department of Animal Husbandry, University of California, Davis, California	Manganese as a key to the pleiotropic action of some pigment mutants
A. C. Fabergé	Department of Zoology, The University of Texas, Austin, Texas	Chromosome and chromatin structure as seen by a new development in electron microscopy
Patricia Farnes	Department of Pathology, Rhode Island Hospital, Providence, Rhode Island	Studies on cytodynamic actions of pokeweed mitogen
John Farrant	Medical Research Council, National Institute for Medical Research, London, England	The problem of organ preservation at low temperatures
Joseph Gally	The Rockefeller University, New York, New York	The problem of the origin of antibody diversity
Norman H. Giles	Yale University, New Haven, Connecticut	Gene clusters coding for enzyme aggregates
Milton P. Gordon	University of Washington, Seattle, Washington	Studies on the photochemistry of TMV and its RNA
Anthony Griffiths	Department of Agronomy, Kansas State University, Manhattan, Kansas	The genetic control of recombination in *Neurospora crassa*

SPEAKER	AFFILIATION	SUBJECT
Klaus Haefner	Division of Biology, Southwest Center for Advanced Studies, Dallas, Texas	UV-induced lethal sectoring in yeast and *E. coli*
Sue Hanlon	Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, Illinois	Polynucleotide conformations in organic solvents
Henry Hellmers	Department of Botany, Duke University, Durham, North Carolina	The phytotron: A tool for botanical research
George Hodgson	The University of Chile, Santiago, Chile	Proliferation state of stem cells, erythropoietin responsive cells, and antigen responsive cells
Miroslav Holub	Division of Immunology, Institute of Biology, Czechoslovakia Academy of Sciences, Prague, Czechoslovakia	Engagement of lymphocytes and histocytes in antibody formation
Sheila Howarth-Thompson	Department of Microbiology, Medical School, University of Otago, Dunedin, New Zealand	The influence of colicine factors on the response to UV-irradiation in enterobacteria
Nino L. Incardono	Department of Chemistry, Florida State University, Tallahassee, Florida	Structural change in ϕX-174 — Its possible implication in the process of adsorption
Pierre Joliot	RIAS, Baltimore, Maryland	Reaction kinetics of photosynthetic oxygen evolution
Warwick E. Kerr	Department of Genetics, University of São Paulo, Ribeirão Prêto, Brazil	Genetics and evolution of bees
Saul Kit	Division of Biochemical Virology, College of Medicine, Baylor University, Texas Medical Center, Houston, Texas	Is the entire SV40 genome integrated in transformed mouse kidney cells?
George Klein	Institute for Tumor Biology, The Karolinska Institute, Stockholm, Sweden	The Burkitt lymphoma and model experiments on mouse leukemia
Margaret Lieb	University of Southern California, School of Medicine, Los Angeles, California	Mapping and complementation studies of mutations in the cI-region of bacteriophage lambda
Knud Karl Lonberg-Holm	E. I. du Pont de Nemours and Co., Central Research Department, Wilmington, Delaware	Nucleic acid synthesis in seedlings
Abraham Mazur	New York Blood Center, New York, New York	Role of the spleen in hemoglobin synthesis in the rat
J. M. Mitchison	Department of Zoology, University of Edinburgh, Edinburgh, Scotland	The relations of enzyme synthesis, DNA synthesis and radiation effects in the cell cycle of *Schizosaccharomyces pombe*

SPEAKER	AFFILIATION	SUBJECT
Fernando Morgado	Institute of Physiology, Southern University of Chile, Valdivia, Chile	Effect of antifolic on the primary and secondary response of mice immunized with rat tissue
Peter W. Neurath	New England Medical Center Hospitals, Boston, Massachusetts	Computer systems for chromosome analysis
Benedetto Nicoletti	Istituto di Genetica Dell' Università, Facolta di Scienze, Citta Universitaria, Rome, Italy	Effects of ultraviolet irradiation on mammalian cells *in vitro*
Alfred Norten	Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee	The structure of water
G. M. Padilla	Wrightsville Marine Biomedical Laboratory, Wilmington, North Carolina	Toxigenesis and growth in marine algae
Roy Patterson	Allergy-Immunology Section, School of Medicine, Northwestern University, Chicago, Illinois	Mechanisms of animal dander and sera sensitization and possible methods of control
Claus Pelling	Max-Planck-Institute for Biology, Tübingen, Germany	Sedimentation properties of RNA prepared from salivary gland cells and of RNA from isolated giant chromosomes
David Penington	The London Hospital, London, England	Regulation of platelet production
E. C. Pollard	Pennsylvania State University, University Park Station, State College, Pennsylvania	The action of ionizing radiation on bacteria
Manfred F. Rajewsky	Max-Planck-Institute for Biophysics, Frankfort, Germany	DNA synthesis and cell proliferation during diethylnitrosamine-induced liver carcinogenesis
Jose Reissig	C. W. Post College, Brookville, L.I., New York	*In vitro* complementation with aspartic transcarbamylase of Neurospora
Richard Roblin	The Biological Laboratories, Harvard University, Cambridge, Massachusetts	5′-Terminal nucleotide sequence studies with phage R17 RNA
Edna M. F. Roe	Department of Biophysical Chemistry, Chester Beatty Institute, London, England	Polysaccharides at cell surfaces: growth inhibition of ascites tumor cells by tragacanth
Arthur Rörsch	Medical-Biological Laboratory RVO-TNO, Rijswijk, The Netherlands	Genetical and biochemical aspects of the breakdown of the alkaloid atropin in *Pseudomonas putida;* an inducible system
Anand Sarabhai	Institute of Molecular Biology, University of Oregon, Eugene, Oregon	Studies on polypeptide reinitiation after chain termination

SPEAKER	AFFILIATION	SUBJECT
Folke Skoog	Department of Botany, University of Wisconsin, Madison, Wisconsin	Cytokinins: Biological effects, sources and structure/activity relationships
R. M. Smillie	CSIRO, Plant Physiology Unit, University of Sydney, Sydney, Australia	Organelle biosynthesis; roles of organelle DNA and protein synthesizing systems
L. A. Stocken	Department of Biochemistry, University of Oxford, Oxford, England	The significance of histones in radiation damage to the nucleus
Benjamin P. Stone	Department of Botany, The University of Tennessee, Knoxville, Tennessee	Effects of actinomycin-D and ionizing radiation upon photosensitive lettuce seed germination
Tsutomu Sugahara	Department of Experimental Radiology, School of Medicine, Kyoto University, Kyoto, Japan	Bone marrow stem cells in relation to radiosensitivity and recovery in mice
Alexander Symeonidis	Thagenion Memorial Cancer Institute, Salonica, Greece	Research activities at the cancer institute in Thessaloniki
Helene W. Toolan	Institute for Medical Research, Putnam Memorial Hospital, Bennington, Vermont	Origin of the H-viruses; a classical incidence of serendipity
Rodes Trautman	Plum Island Animal Disease Laboratory, United States Department of Agriculture, Greenport, L.I., New York	The impact of computers on ultracentrifugation
J. J. Trentin	Baylor Medical School, Baylor University, Waco, Texas	Results of caesarean derivation and barrier maintenance of a large inbred mouse colony
Johannas van Overbeek	Director, Institute of Life Sciences, Texas A & M University, College Station, Texas	Dormin, a new plant hormone
Albert J. Wahba	Department of Biochemistry, New York University School of Medicine, New York, New York	Translation of the genetic message: Chain initiation and termination in *E. coli*
Jon Weil	Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee	Recombination deficient mutants of bacteriophage lambda
Edward Westhead	Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts	Feedback inhibition of two apartyl kinases from *E. coli*
Rolf Wideröe	Brown Boveri Company, Baden, Switzerland	Radiobiology and radiotherapy
Hans Wigzell	Department of Tumor Biology, The Karolinska Institute, Stockholm, Sweden	Studies on induction and inhibition of antibody synthesis
G. Congdon Wood	American Cancer Society, Inc., New York, New York	New development in the research program of the American Cancer Society

SPEAKER	AFFILIATION	SUBJECT
Arthur M. Zimmerman	Department of Zoology, University of Toronto, Toronto, Canada	Macromolecular synthesis in *Tetrahymena pyriformis:* A high pressure study

UNIVERSITY OF TENNESSEE–OAK RIDGE GRADUATE SCHOOL OF BIOMEDICAL SCIENCES – BIOLOGY DIVISION SPECIAL LECTURE SERIES

In the fall of 1967, a special joint lecture series was begun under the joint sponsorship of the University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences and the Biology Division. There were two speakers under this program during November:

SPEAKER	AFFILIATION	SUBJECT
H. W. Wycoff	Department of Molecular Biophysics, Yale University, New Haven, Connecticut	The three dimensional structure, chemistry, and genetics of ribonuclease
E. R. Stadtman	Laboratory of Biochemistry, National Heart Institute, Bethesda, Maryland	The regulation of glutamine synthetase in *Escherichia coli*

SCHEDULED SPEAKERS AT PROFESSIONAL MEETINGS IN 1968

Papers by members of the Biology Division will be presented at the following professional society meetings (listed in chronological order) during 1968. Names of speakers appear in boldface. (Listing includes information received up to February 15, 1968.)

Symposium on Modern Separation Methods of Macromolecules and Particles, Madison, Wisconsin – **N. G. Anderson**

Twelfth Annual Meeting of the Biophysical Society, Pittsburgh, Pennsylvania – **M. E. Boling,** Sohan Modak, Gerald Price, Alice Mattingly, M. P. Gordon, and Jane K. Setlow; **J. E. Donnellan, Jr.,** R. S. Stafford, J. L. Hosszu, and R. O. Rahn; **Barbara A. Hamkalo** and P. A. Swenson; **B. E. Moseley;** **R. O. Rahn,** Jane K. Setlow, and J. L. Hosszu; **Jane K. Setlow,** Dianne C. Brown, and M. E. Boling; **R. B. Setlow** and W. L. Carrier; **P. A. Swenson;** and **J. J. ten Bosch,** R. O. Rahn, and J. W. Longworth

Florida State University Alumni Studies Conference on Molecular Biology, Tallahassee – **W. E. Barnett** and J. L. Epler; **J. L. Epler** and W. E. Barnett; and **J. D. Regan**

155th National Meeting of the American Chemical Society, San Francisco, California – **W. E. Cohn;** **J. X. Khym**

American Association of Anatomists, New Orleans, Louisiana – **J. N. Dumont**

59th Annual Meeting of the American Association for Cancer Research, Atlantic City, New Jersey – **C. T. Bahner** and D. G. Doherty; **N. K. Clapp** and A. W. Craig; **Stanfield Rogers;** and **H. E. Walburg, Jr.,** and G. E. Cosgrove

Federation of American Societies for Experimental Biology, Atlantic City, New Jersey – **N. G. Anderson**, C. E. Nunley, and C. T. Rankin, Jr.; **D. G. Doherty** and Jesse James; **R. K. Fujimura** and Elliot Volkin; **Joan Wright Goodman** and Sarah G. Shinpock; **D. L. Groves**; **C. B. Hager**; **W. W. Harris** and Frances L. Ball; **F. C. Hartman**; **Dona C. Hooper** and J. F. Albright; **K. B. Jacobson**, J. A. Knopp, and J. B. Murphy; **V. K. Jenkins**, A. C. Upton, and T. T. Odell, Jr.; **A. D. Kelmers**, R. L. Pearson, L. R. Shugart, and J. F. Weiss; **T. J. Mitchell**, C. C Congdon, M. A. Kastenbaum, and D. A. Gardiner; **Peter Pfuderer**; **T. T. Odell, Jr.**, C. W. Jackson, and R. S. Reiter; **B. J. Ortwerth**, Ugo Del Monte, Lawrence Rosen, and G. David Novelli; **J. R. Reel** and F. T. Kenney; **W. S. Riggsby**; **Lee Shugart**, G. David Novelli, and M. P. Stulberg; **Mayo Uziel** and H. G. Gassen; **W. K. Yang**, G. David Novelli, and C. J. Wust

Southern Section, American Society of Plant Physiologists, Athens, Georgia – **D. E. Foard**; **A. H. Haber** and L. L. Triplett; **D. H. O'Dell** and D. E. Foard

Association of Southeastern Biologists, Athens, Georgia – **P. W. Whiting**

Tennessee Society of Pathologists and Tennessee Medical Association, Chattanooga – **R. C. Brown** and H. C. Hopps

16th Annual Meeting of the Radiation Research Society – **N. K. Clapp**, E. B. Darden, Jr., R. S. Bender, and M. C. Jernigan; **E. B. Darden, Jr.**, K. W. Christenberry, M. C. Jernigan, and J. W. Conklin; **F. J. de Serres**; **F. J. de Serres** and B. B. Webber; **Mary Esther Gaulden**; **J. L. Montour**, E. B. Darden, Jr., and A. C. Upton; **M. L. Randolph**; **J. D. Regan** and Susumu Takeda; **R. C. von Borstel**, R. H. Smith, D. S. Grosch, Anna R. Whiting, R. L. Amy, and Sohei Kondo; **H. E. Walburg, Jr.**, and Edna I. Mynatt

Symposium on Dose Rate in Mammalian Radiation Biology, Oak Ridge, Tennessee – **A. C. Upton**

American Society for Microbiology, Detroit, Michigan – **Roy Curtiss III** and D. R. Stallions

19th Annual Meeting of the Tissue Culture Association, San Juan, Puerto Rico – **R. B. Cumming**; **J. D. Regan**, J. S. Cook, and Susumu Takeda; and **R. L. Tyndall**, N. D. Bowles, J. A. Otten, and A. C. Upton

IIIrd International Congress of Endocrinology, Mexico City, Mexico – **J. R. Reel**

IIIrd International Congress of Histochemistry and Cytochemistry, New York City – **R. C. Allen** and Dorothy J. Moore

12th International Congress of Genetics, Tokyo, Japan – **U. H. Ehling**, D. G. Doherty, and H. V. Malling; **E. H. Y. Chu** and H. V. Malling; and **H. V. Malling** and F. J. de Serres

Fifth International Photobiology Congress, Hanover, New Hampshire – **J. S. Cook** and A. J. Zilnis; **A. H. Haber**, P. J. Thompson, P. L. Walne, and L. L. Triplett; **A. P. Harrison, Jr.**; and **J. D. Regan** and J. S. Cook

12th Congress of the International Society of Hematology, New York City – **T. T. Odell, Jr.**, C. W. Jackson, Rebecca S. Reiter, and D. Charsha

Foreign Travel

During the period February 1–December 31, 1967, more than 40 members of the Biology Division traveled to foreign countries to give invited talks at colleges, universities, and research institutes; to confer with colleagues; and to participate in conferences and meetings. Countries visited were:

Europe – Austria
Belgium
Czechoslovakia
England
France
Germany
Greece
Italy
Romania
Scotland
Sweden
Switzerland

Asia – India
Israel
Japan
Pakistan
Thailand

Australia – Commonwealth of Australia

The Americas – Argentina
Brazil
Canada
Chile
Mexico
Peru
Venezuela

H. I. Adler traveled in Italy, Greece, and Israel during May and June. In Italy, Dr. Adler spoke on "Radiation sensitivity of bacteria" at the Casaccia Nuclear Center, and in Greece, at the Thessaloniki Cancer Institute, his subject was "Genetic control of cell division in bacteria." Dr. Adler concluded his foreign travel by participating as a guest lecturer in a training course held at the Soreq Nuclear Center near Tel-Aviv, Israel.

J. F. Albright was in Buenos Aires, Argentina, August 31–September 18, to instruct in an international training course in immunology for postgraduate students from various South American countries (see **Educational Activities** for details). Dr. Albright also participated in the Symposium on Haemopoietic Stem Cell Kinetics, held in Manchester, England, October 9–11, and visited biological laboratories in Mill Hill and Leeds before returning to the Division on October 16.

R. C. Allen was in Toronto, Canada, December 2–5, for the 10th annual meeting of the American Society of Hematology. He gave a paper at the meeting entitled "Further purification of mouse plasma erythropoietin and the effects of its presence on plasma esterase isoenzymes."

N. G. Anderson was at the Chester Beatty Institute, London, England, to confer with Dr. Peter Alexander and at the University of Sussex to confer with E. J. Ambrose. From England, Dr. Anderson traveled to Prague, Czechoslovakia, to attend the Tenth International Congress of Microbiological Standardization. He returned to England to discuss problems of centrifugation with S. P. Spragg, University of Birmingham, Birmingham; J. C. Z. Martin, Measuring and Scientific Equipment, Inc., London; and A. P. Matthias, University College, London.

J. G. Brewen, while on a year's leave of absence at the CSIRO, Canberra, Australia (see **Division Members Working in Other Laboratories** for details), attended the ANZAAS – Genetical Society Cytogenetics Symposium held in Melbourne, Australia, January 16, 1967, where he presented a paper entitled "Sister chromatid exchanges: Are they spontaneous or induced?" During his stay in Australia, Dr. Brewen also gave two seminars at the University of Adelaide, Adelaide, and attended the International Conference on Replication and Recombination of Genetic Material, held in Canberra, August 28–September 1.

L. G. Caro, while on leave in England from summer 1966 to spring 1967 (see **Division Members Working in Other Laboratories** for details), visited a number of universities and research institutes in the

British Isles and continental Europe to give seminars and to confer with staff scientists on problems of mutual concern. He visited the Institute of Molecular Biology, Geneva, Switzerland, September 28–October 4, 1966, to confer with Professor Edouard Kellenberger on the organization of a course in electron microscopy and to give a seminar. In October and November he was at the Universities of Edinburgh (Scotland), Glasgow (Scotland), and Leicester (England), at each of which he presented the seminar, "Role of F-pili in bacterial conjugation." In January, 1967, he traveled to Brighton, England, to give a seminar at the University of Sussex. From March 8 to 11, Dr. Caro was at the Pasteur Institute in Paris, France, to confer with colleagues and present a seminar; before returning to London, he made a brief visit to Geneva.

Dr. Caro revisited Europe from August 23 to October 1, having returned to the Division in April. First he traveled to Geneva, Switzerland, to instruct in the electron microscopy course mentioned above (see **Educational Activities** for details). Then he went to the Laboratory of Genetics, Toulouse, France, for discussions with Drs. J. M. Louam and Michel Sicard; then to the Microbial Genetics Research Unit, MRC, London, England, for discussions with Drs. Claire M. Berg and William Hayes.

E. H. Y. Chu did collaborative research on tumor immunology at the Karolinska Institute, Stockholm, Sweden, in January and February (see **Division Members Working in Other Laboratories** for details).

W. E. Cohn attended the annual meeting of the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry, held at Ravello, Italy, April 11–16. On August 18, Dr. Cohn was in Tokyo, Japan, for the Seventh International Congress of Biochemistry, after which he visited and consulted with colleagues at the Tokugawa Institute, the University of Tokyo, and the Tokyo Medical and Dental University.

C. C Congdon was in Paris, France, June 26–30, to attend a bone marrow conference and the First International Congress of the Transplantation Society. Dr. Congdon visited the Institute of Oncology in Bucharest, Romania, before returning to the Division.

M. I. Dolin, R. K. Fujimura, R. C. Fuller, K. Bruce Jacobson, F. T. Kenney, J. X. Khym, G. David Novelli, and M. P. Stulberg were in Tokyo, Japan, August 19–25, to present papers at the Seventh International Congress of Biochemistry. Also attending the meeting were W. E. Cohn, B. J. Ortwerth, and Mayo Uziel. Dr. Cohn served as chairman of a symposium, and Dr. Novelli was chairman of a session.

M. I. Dolin, prior to the Tokyo Congress, attended a meeting on Flavins and Flavoproteins in Nagoya, August 14–17.

James Dumont presented a seminar to the Zoology Department of the University of Windsor, Windsor, Ontario, Canada, November 28–30. The title of his talk was "Electron microscopic study of oocyte development and yolk formation in the horse crab, *Limulus polyphemus*."

R. K. Fujimura consulted with colleagues at Osaka University and the University of Tokyo after participating in the Biochemistry Congress.

R. C. Fuller, before presenting a paper at the Tokyo Biochemistry Congress, chaired a session of the Symposium on Comparative Biochemistry and Biophysics of Photosynthesis, Hakone, Japan, August 16–18. Dr. Fuller was co-organizer of the symposium.

Joan Wright Goodman attended a one-day bone marrow conference in Paris, France, on June 26, and gave a paper at the First International Congress of the Transplantation Society, also held in Paris, June 26–30. The title of her presentation was "Factors that modify poor growth of parental cells in F_1 hybrid mice."

E. H. Grell traveled to Germany in February to attend a conference on Genetics, Biochemistry, and Physiology of Insect Eye Color Mutants, held in Munich, March 2–4.

Alexander Hollaender, on January 4, 1967, began a foreign tour which took him to France, Switzerland, Greece, Thailand, Pakistan, India, Israel, and Italy. He went first to Paris, France, where he met with Dr. Carlos Chagas, Brazilian Permanent Delegate to UNESCO, to discuss the role of UNESCO in Latin America, especially Brazil. From Paris, he traveled to Geneva, Switzerland, to discuss a planning institute in the biomedical sciences with Dr. Martin M. Kaplan of WHO. Then he went to Basle for discussions at F. Hoffman-La Roche and Co. on new developments in pharmacological genetics and to give a seminar on "Studies going on in the Biology Division, Oak Ridge National Laboratory." On January 11, Dr.

Hollaender arrived in Athens, Greece, for talks with Dr. C. B. Krimbas of the Department of Genetics, College of Agriculture, concerning the program for a possible symposium on genetic polymorphism to be held in Athens in 1968. He then visited the University of Athens School of Medicine and next made an invited survey of the "Democritos" Nuclear Research Center. On January 16, Dr. Hollaender visited the Cancer Institute, Theagenion Memorial, in Thessaloniki, Greece, to survey the biology program there and to give a lecture on Biology Division activities. From January 19 to March 9, Dr. Hollaender visited various research institutions and universities in Thailand, Pakistan, India, and Israel, at which he reviewed biology programs and made suggestions to help bring them into the mainstream of modern biology. In addition, he gave seminars on "Work at the Biology Division, Oak Ridge National Laboratory" at the Siriraj Medical School, Bangkok, Thailand; at the University of Dacca and the Jute Institute, Dacca, Pakistan; and, in India, at the University of Chandigarh, Chandigarh, the University of Rajasthan, Jaipur, and to the Biology Group at the Bhabha Atomic Research Center, Bombay. Dr. Hollaender then returned to Europe to spend the final week of his trip, March 10–16, in Rome, Italy, at the Comitato Nazionale per l'Energia Nucleare (CNEN) discussing current research problems in radiobiology in his capacity as consultant to CNEN.

Dr. Hollaender spent the first half of July in Brazil and Peru. July 2–3, he was at the University of São Paulo, São Paulo, Brazil, to review the present biology program there and to discuss possible cooperation between the University and the Biology Division. On July 4, he met with staff members of the Institute of Biology at the University of Campinas, Campinas, Brazil. From July 5 to 8, he was in Belo Horizonte, Brazil, to make final plans for the International Symposium on Nuclear Physiology and Differentation to be held there December 1–6, 1968. From Belo Horizonte, Dr. Hollaender traveled to Rio de Janeiro to attend the Symposium on the Peaceful Uses of Atomic Radiation, at which he presented the opening address and served as chairman of one of the sessions. Finally, he went to Lima, Peru, where he visited the University of San Marcos to help in preparations for a modern biology course to be given by Brazilian scientists at San Marcos University in cooperation with ORNL.

In November, Dr. Hollaender returned to South America, arriving in São Paulo, Brazil, on the 25th for discussions with several of his colleagues. From São Paulo, he traveled to Recife to visit the University of Pernambuco and to discuss with staff members the possibilities for cooperation between the University and the Biology Division. Dr. Hollaender went next to Fortaleza, where he visited various departments of the University of Ceara – then to Rio de Janeiro, where he toured the Biophysics Institute of the University of Brazil and conferred with members of the staff, particularly Dr. L. R. Caldas and Dr. Aristides Pachoco Leao. While in Rio, he met with Dr. Antonio Couseiro and Dr. Manoel da Frota Moroira, both of the National Research Council, to discuss the scientific development of Brazil. On December 3, Dr. Hollaender left Brazil to participate in the International Symposium on Basic Mechanisms in Photochemistry and Photobiology, held in Caracas, Venezuela, December 4–8. He returned to the Division on December 10.

K. Bruce Jacobson was in Osaka and Nagoya, Japan, August 14–19, to confer with colleagues at various biological laboratories, before going to Tokyo to attend an informal Symposium on Nucleic Acids and Protein Synthesis and to participate in the Biochemistry Congress.

F. T. Kenney visited laboratories in Tokyo, Tokushima, and Osaka, Japan, August 9–11 and 14–15 for discussions with staff scientists on research problems of mutual interest. Afterwards, he presented a paper at the Fifth Gunma Symposium on Endocrinology, Kusatusu Spa, Japan, August 18–19, and then attended the Biochemistry Congress in Tokyo.

J. X. Khym, after presenting a paper at the Biochemistry Congress in Tokyo, August 19–25, spoke on "Determination of sequence in nucleic acids" at Takeda Chemical Industries, Ltd., Osaka, Japan, August 29. He also visited Osaka University.

S. P. Leibo spent August 28–December 11 in Geneva, Switzerland, at the Institute of Molecular Biology, University of Geneva, where he conducted collaborative research with Dr. Edouard Kellenberger. While there, Dr. Leibo attended a course on Applications of Electron Microscopy to Molecular Biology sponsored by the European Molecular Biology Union (see **Educational Activities** for details). Dr. Leibo spoke at both the National Institute for Medical Research, Mill Hill, London, England, and the University of Cambridge before returning to the Division on December 15.

D. L. Lindsley attended the Conference on Replication and Recombination of Genetic Materials, Canberra, Australia, August 27–31, and presented a paper entitled "The genetic control of recombination in *Drosophila*."

J. W. Longworth attended the International Symposium on Basic Mechanisms in Photochemistry and Photobiology, held December 4–8 in Caracas, Venezuela, and spoke on "Excited state interactions in macromolecules."

Takashi Makinodan was in South America from September 12 to October 21. He participated in the International Training Course in Immunology held in Buenos Aires, Argentina (see **Educational Activities** for details); delivered lectures to the Medical Faculty of the University of Chile and to the Chilean Society of Genetics, Santiago, Chile; and consulted on problems of mutual interest with various research groups at the University of Chile, Santiago; the Southern University of Chile, Valdivia; and the Institutes of High Altitude and Cancer Research of the University of Cayetano Heredia, Lima, Peru.

H. V. Malling spent May 20–22 at the Department of Human Genetics and Anthropology, University of Freiburg, Germany, where he discussed with various investigators the problem of chemical mutagens as a possible genetic hazard.

G. David Novelli, in conjunction with his travel to Tokyo, Japan, for the Biochemistry Congress, visited the Laboratory of Molecular Biology of the National Cancer Institute of Tokyo, and the Division of Molecular Biology, Institute of Cancer Research, Kanazawa.

E. F. Oakberg attended an IAEA Study Group Meeting on the Effects of Ionizing Radiations on Meiotic Systems, Vienna, Austria, May 8–11. He was a co-chairman of the meeting.

John Papaconstantinou was at the University of Ghent, Ghent, Belgium, June 5–9, to attend the Symposium on Lens Proteins and Related Subjects and to present a paper on "The regulation of protein and nucleic acid synthesis during cellular maturation." Later, June 9–16, he visited with Drs. A. Symeonides and G. Georgatsos in Thessaloniki, Greece.

R. O. Rahn attended and gave a paper ("The photochemistry of DNA at 77°K") at the International Symposium on Basic Mechanisms in Photochemistry and Photobiology, held in Caracas, Venezuela, December 4–8.

J. D. Regan spent September 4–18 in Germany. During this period, he presented a seminar, entitled "Reparatur von UV-induzierter Schäden in Zellen in Vitro," at both the Institut für Strahlenbiologie, Karlsruhe, and the Institut für Theropeutische Biochemie, Frankfurt, and conferred with staff members at the Max-Planck-Institut für Virusforschung, Tübingen.

Stanfield Rogers was in Lausanne, Switzerland, July 11–13, to confer with Dr. Roger Weil of the Swiss Institute for Experimental Cancer Research. July 17–19, he conferred with Drs. Breitburd and Orth at the Pasteur Institute, Paris, France, and July 20–26 he was at the National Institute of Medical Research, London, England, to confer with Drs. Peter Medawar and Alexander Haddow.

Liane B. Russell attended the Fourth Basel Colloquium on Mammalian Sex Chromosomes in Differentiation and Development, Basel, Switzerland, March 29–April 1, and presented a paper entitled "Re-evaluation of the Lyon hypothesis on the basis of evidence from classical genetic markers." In May, she traveled to Vienna, Austria, for an IAEA Study Group Meeting on the Effects of Ionizing Radiations on Meiotic Systems, where she spoke on "The use of sex-chromosome aberrations for measuring radiation effects in mammals."

W. L. Russell visited various biological laboratories in Brazil during July, and gave an invited paper at the Symposium on the Pacific Uses of Atomic Radiation, sponsored by the Brazilian Academy of Science and held at Rio de Janeiro.

R. B. Setlow went to Javne, Israel, in May and lectured to members of an IAEA training course held at the Soreq Nuclear Research Center. August 27–September 1, he attended the International Conference on Replication and Recombination of Genetic Material, Canberra, Australia, where he spoke on "The excision of pyrimidine dimers *in vivo* and *in vitro*." Afterwards, he visited the University of Melbourne and gave a seminar on "Photochemistry of polynucleotides." He then met with G. W. Grigg, Division of Animal Genetics of CSIRO in Sydney. He concluded his trip with a visit to Pakistan, where he conferred with Amir Muhammed of the Atomic Energy Center and presented a series of lectures on photochemistry

and photobiology of polynucleotides and a single lecture entitled "Modern trends in biology" at both the Atomic Energy Center and the Departments of Botany and Zoology, University of Punjab. December 4–8, Dr. Setlow was in Caracas, Venezuela, for the International Symposium on Basic Mechanisms in Photochemistry and Photobiology at which he gave a paper entitled "Photoproducts in DNA irradiated *in vivo*."

M. L. Simmons was in Nagoya, Japan, April 6–9, to attend the International Symposium on Gnotobiotic Life in Medical and Biological Research and to give a paper on "Specific pathogen-free rat production in plastic germfree isolator rooms." While in Nagoya, Dr. Simmons visited the germfree laboratories of the University of Nagoya. Before returning to the Division, he also visited the Biology, Zoology, Radiation Biology, and Biochemistry Departments of the University of Kyoto, Kyoto, Japan.

M. P. Stulberg had a full itinerary of travel while in Japan for the Biochemistry Congress held in Tokyo during August. He was first in Kanazawa for consultations with T. Kameyama of the University of Kanazawa, and then at Kyoto for talks with Y. Kawade at Kyoto University. After the Congress, Dr. Stulberg visited the National Cancer Center Research Institute, Tokyo, to talk with S. Nishimura, and the Tokugawa Institute for Biological Research, Tokyo, for talks with T. Iwamura. He presented lectures at Kanazawa and the National Cancer Center.

A. C. Upton went to England in April to attend a meeting of the International Commission on Radiological Protection and of its committees. During June and July, Dr. Upton traveled in Peru and Brazil, first going to Lima for collaborative research and to give a seminar at the Peruvian Society of Oncology. Later, he discussed radiation and cancer research with the faculties of Radiology, Pathology, and Experimental Biology in the Universities of São Paulo, Brasilia, and Bahia in Brazil. At the University of São Paulo School of Medicine at Ribeirão Prêto, he discussed radiation and cancer research. Finally, he participated in a symposium sponsored by the Brazilian Academy of Sciences and attended a meeting of the Brazilian Association for the Advancement of Sciences. July 24–25, Dr. Upton was in Harwell, England, for a meeting of the International Commission on Radiological Protection Task Group on the Radiosensitivity of Different Tissues in Relation to Radiation Protection.

Mayo Uziel spoke on "Structure of transfer RNA" to the Faculty of Medicine at the University of British Columbia, Vancouver, Canada, in August.

R. C. von Borstel presented a paper entitled "Dicentric translocations as a component of dominant lethality in irradiated Habrobracon sperm" at the International Chromosome Conference in Oxford, England, September 5–8. He then conferred with officials of the International Atomic Energy Agency in Vienna, Austria, on programs of radiobiological interest in the Middle and Far East. From Austria he went to Athens, Greece, for a discussion with C. B. Krimbas of the College of Agriculture and S. Georgopoulous of the "Democritus" Nuclear Center concerning the program for a symposium on Genetic Polymorphism being planned for 1969. At the Center he advised on the control of the olive fly, Dacus, by the irradiation-of-male method. During mid-September, Dr. von Borstel visited and lectured at Pakistani Atomic Energy Centers in Karachi and Pinstech; at the Centers in Tandojam, Lahore, and Dacca; and at the Universities of Dacca and Lahore. He also conducted a short course on Chromosome Structure and Function at the Atomic Energy Center in Lahore.

On September 23, Dr. von Borstel went to Bangkok, Thailand, where he consulted with officials at the U.S. Embassy, and advised on pest control and lectured at the Thailand Office of Atomic Energy for Peace. He also lectured at Siriraj Medical School in Bangkok. On September 26, he joined a task force arranged by the Southeast Asia Ministers of Education Secretariat to study Indonesian proposals for establishing three regional centers in Bogor. While in Indonesia he consulted with Dr. O. Soemarwoto, the director of the National Biological Institute, on the possibility of arranging an international symposium on Tropical Molecular Biology. He also presented lectures at the Institute in Bogor, the Medical College of the University of Indonesia in Djakarta, and the Institute of Technology in Bandung.

On October 1, Dr. von Borstel went to Bangalore, India, to visit the Indian Institute of Science to discuss regional education problems and to present a seminar on "Super-suppressors." From there he traveled to Bombay to discuss radiobiological problems with Dr. Gopal-Ayengar and his colleagues at the Bhabha Atomic Research Center; he presented seminars there and at the Tata Institute. He visited with biologists at Aurangabad and presented lectures in New Delhi at the University and the Pusa Institute. He also obtained different species of Habrobracon for the genetic collections in the United States.

He spent October 14–20 in Naples, where he attended the annual meeting of the Italian Genetics Association and discussed genetic research at the International Laboratory of Genetics and Biophysics, particularly with Drs. F. M. Ritossa, E. Calef, and A. A. Buzzati-Traverso.

Dr. von Borstel returned to Europe in early December to attend the Symposium on the Use of Isotopes and Radiation in Entomology held in Vienna, Austria; to confer with Dr. Herbert Marcovich of the Radium Institute, Paris, France; and to meet with Dr. Giovanni E. Magni in Parma, Italy, to discuss research in yeast genetics and radiobiology.

G. L. Whitson traveled to Canada on June 6 to attend the Society of Protozoologists meetings in Toronto and to present a paper entitled "Production of thymine dimers and repair in ultraviolet-irradiated *Tetrahymena pyriformis*, strain GL-C." From Toronto, Dr. Whitson went to Montreal to confer with staff members of the Department of Zoology, McGill University.

Tuneo Yamada attended the Ciba Foundation Symposium on Cell Differentiation held in London, England, January 31–February 2, and gave a paper on "Cellular synthetic activities in induction of tissue transformation." Afterwards, he visited Prof. Etienne Wolff at the Laboratory of Experimental Embryology, College of France, Nugent-sur-Marne, France; participated in an executive committee meeting of the International Institute of Embryology at the College of France; and held a seminar, "Sequential changes in cellular synthetic activities associated with tissue transformation," at the Institute of Physiochemical Biology, Paris, after visiting Dr. Kraus Scherrer of the Institute. He returned to the Division on February 7.

Dr. Yamada was in Interlaken, Switzerland, September 4–8, for the VIIIth International Embryological Conference and the Board Meeting of the International Institute of Embryology. At the conference he gave a demonstration on "Cellular synthetic activities in lens formation," and presented reports at two of the discussion groups. In mid-December, Dr. Yamada lectured on "Control of cellular differentiation" at the University of Toronto, Toronto, Ontario, Canada. His talk was sponsored by the University's Department of Zoology.

Meetings and Conferences

1967

PROCEEDINGS OF TWENTIETH ANNUAL BIOLOGY RESEARCH CONFERENCE

The proceedings of the twentieth annual Biology Research Conference (**Chromosome Mechanics at the Molecular Level**), held at Gatlinburg, Tennessee, April 10–13, 1967, were published as a supplement to the October issue of the *Journal of Cellular Physiology*. Listed below is the table of contents:

R. F. Kimball	Introduction
Franklin W. Stahl	Circular genetic maps
C. A. Thomas, Jr.	The rule of the ring
S. Spiegelman, I. Haruna, N. R. Pace, D. R. Mills, D. H. L. Bishop, J. R. Claybrook, and R. Peterson	Studies in the replication of viral RNA

John Cairns and Cedric I. Davern	The mechanics of DNA replication in bacteria
François Cuzin, Gérard Buttin, and François Jacob	On the mechanism of genetic transfer during conjugation of *Escherichia coli*
Kwen-Sheng Chiang and Noboru Sueoka	Replication of chromosomal and cytoplasmic DNA during mitosis and meiosis in the eucaryote *Chlamydomonas reinhardi*
Matthew Meselson	Reciprocal recombination in prophage lambda
Rhoda F. Grell	Pairing at the chromosomal level
A. H. Doermann and David H. Parma	Recombination in bacteriophage T4
A. J. Clark	The beginning of a genetic analysis of recombination proficiency
Jerard Hurwitz, Andrew Becker, M. L. Gefter, and M. Gold	Enzymatic reactions at termini of DNA
Jun-ichi Tomizawa	Molecular mechanisms of genetic recombination in bacteriophage. Joint molecules and their conversion to recombinant molecules
Rollin D. Hotchkiss	Perspectives

SECOND INTERNATIONAL CONFERENCE ON CELL SYNCHRONY

The **Second International Conference on Cell Synchrony,** held at the Biology Division, Oak Ridge National Laboratory, April 27–29, was attended by some 130 investigators from Canada, England, Scotland, Germany, Japan, Russia, and the United States. The meeting dealt with recent advances in the field.

The program, sponsored jointly by the Biology Division and Duke University, was divided into five sessions: (1) Genetic studies in cell synchrony, (2) Developmental aspects of cell synchrony, (3) Regulation and control in synchronized cells, (4) Biochemistry and physiology of synchronized cells, and (5) Macromolecular aspects of cell division synchrony. More than 30 papers were presented, and discussions were interspersed throughout the program.

Alexander Hollaender was chairman. G. M. Padilla (Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina) and G. L. Whitson (Biology Division) were co-chairmen of the organizing committee. Other members were W. D. Fisher (Biology Division) and I. L. Cameron (Department of Anatomy, Upstate Medical Center, State University of New York, Syracuse, New York).

AEC-NCI COCARCINOGENESIS REVIEW

A group of 26 scientists representing the National Institutes of Health (NIH) and the U.S. Atomic Energy Commission (AEC) visited the Biology Division June 8 to review the AEC-NCI Basic Cocarcinogenesis Program.

Visitors were Creed Abell, Carl Baker, Nathaniel Berlin, Howard Bond, Vincent Bono, Jr., Charles Boone, Mortimer Elkind, Melvin Fish, Benjamin D. Fremming, Carl Hansen, James Kieley, Paul Kotin, Frank E. Lundin, Jr., Robert Manaker, Tim O'Connor, William Payne, Herbert Rapp, Frank Rauscher, Pauline Stephan, Robert Stevenson, and William Walter of the NIH; W. W. Burr, Jr., and Robert Rabson of the AEC; Emmanuel Farber, University of Pittsburgh; Joseph L. Melnick, Baylor University; and Gerald Mueller, University of Wisconsin Medical School.

An abstract booklet covering some of the cocarcinogenesis projects was prepared for the visiting officials. In addition, ten Division scientists discussed their work. The program follows:

G. David Novelli	Studies on transfer RNA during ethionine carcinogenesis
F. T. Kenney	Regulation of enzyme synthesis and degradation in liver and hepatoma
Stanfield Rogers	Concerning virus coded genetic information and its manipulation
F. J. de Serres	The relation between carcinogenicity and mutagenicity
E. H. Y. Chu	Mutations in cultured mammalian cells
D. G. Doherty	Inhalation Carcinogenesis Program: a. Method
Paul Nettesheim	Inhalation Carcinogenesis Program: b. Pathology
J. R. Einstein	The x-ray crystallography project for structure determination of large molecules
A. C. Upton	Mammalian chemical carcinogenesis and cocarcinogenesis
N. G. Anderson	Review of the Molecular Anatomy Program

THIRD CONFERENCE ON BLOOD PLATELETS

More than 35 reports were given at the **Third Conference on Blood Platelets** held June 22–23 at the Oak Ridge Playhouse. Organized by T. T. Odell, Jr., Biology Division, and R. M. Heyssel, Vanderbilt University Medical School, the meeting provided an opportunity for investigators to discuss work on the functions and characteristics of blood platelets.

The meeting was supported by the Biology Division, Oak Ridge National Laboratory. Previous meetings in the series were held in 1962 and 1964.

EIGHTH ANNUAL SYMPOSIUM OF THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

Cytodifferentiation with Special Reference to Immunology was the subject of the **Eighth Annual Symposium of the International Society for Cell Biology** held October 9–13 in Gatlinburg, Tennessee. Takashi Makinodan and John Papaconstantinou assisted in planning the meeting and in making arrangements for participants to visit with Biology Division staff members in their laboratories in Oak Ridge. There were nine half-day sessions during which some 25 papers were presented on the general topics "Use of immunologic techniques (indicator assay)" and "Immunologic response as a model system to study differentiation."

CENTRIFUGAL TECHNIQUES FOR VACCINE PURIFICATION

More than 75 scientists attended a meeting on **Centrifugal Techniques for Vaccine Purification,** held October 23–24 at the Oak Ridge National Laboratory, to discuss the use of zonal centrifuges for virus isolation and purification.

Ten papers were presented, covering biological, clinical, and engineering data of vaccine purification such as influenza vaccine and isolation of RS and leukemia virus.

Drawings of the systems most applicable to vaccine production – B-XIV, XV, and K-II – were released to centrifuge manufacturers.

The meeting was sponsored by the Molecular Anatomy Program, with J. L. Liverman and N. G. Anderson as conference chairmen.

VIRAL, TUMOR, AND TRANSPLANTATION ANTIGEN ISOLATION

A working **Conference on Viral, Tumor, and Transplantation Antigen Isolation** was held October 25–27 at the Oak Ridge National Laboratory. More than 75 scientists attended to informally discuss the problem of biophysical and biochemical characterization of such antigens.

Thirty-two papers were presented, covering various methods for isolation and assay of virally induced tumor antigens and naturally occurring transplantation antigens.

The meeting was sponsored by the Biology Division. J. L. Liverman was conference chairman, and N. G. Anderson, R. A. Popp, and William Boyle served on the organizing committee.

HABROBRACON-MORMONIELLA CONFERENCE

The **Habrobracon-Mormoniella Conference** was held October 27–28 at the Biology Division. The program, which was dedicated to Phineas W. Whiting on the occasion of his 80th birthday, was arranged by Roger H. Smith and R. C. von Borstel.

Dr. Whiting, a Biology Division consultant since 1956, pioneered the use of the parasitic wasp Habrobracon for studies in mutation genetics. From 1917 to 1919, he and co-workers bred thousands of Habrobracon generations before finally finding and isolating the historic mutant that produced offspring with orange eyes instead of the normal black. In 1927 he was among the first to apply Muller's technique with x rays, increasing manyfold the rate both of visible and of lethal mutations. He was the first to show that neutrons induced dominant lethals. Chromosomes were mapped, aided here also by haploidy of the males. His work revealing the method of sex determination in Habrobracon was later applied to the honeybee, and this proved of practical value, doubling the fecundity of queen bees when tested stocks were used.

By crossing different species of a second genus of parasitic wasp, Melittobia, he showed that the fertilized eggs, potential females, failed to develop, while the unfertilized were developing parthenogenetically into males of the maternal species.

Since 1949 he has been working with another wasp, Mormoniella, in which the method of sex determination is not understood. This wasp has an unusually high mutation rate for scarlet and white eye-color genes, making it possible to study multiple associated effects causing sterility and inviability.

Dr. Whiting taught at various schools from 1918 until retirement in 1957 from his position as professor at the University of Pennsylvania. Since then, he has devoted his time to research and consulting responsibilities. About half of the conference's 40 participants were scientists who had been taught by Dr. Whiting or by one of his students.

The conference dealt with recent advances in the field. General topics covered were: Radiation and chemical mutagenesis, Cytology, Gene action, Extrachromosomal inheritance, Gene transmission, Genetic nomenclature, and Macro- and microgenetic mapping. Dr. R. F. Kimball presented the welcome address. Dr. von Borstel gave the introduction.

ANNUAL INFORMATION MEETING

The Oak Ridge National Laboratory Advisory Committee for Biology made its annual visit to the Division December 4 to 6. The committee is composed of C. E. Carter, Western Reserve University Medical School; Rollin D. Hotchkiss, Rockefeller University; Henry S. Kaplan, Stanford University Medical School; Herschel Roman, University of Washington; Earl R. Stadtman, National Heart Institute; and Curt Stern, University of California.

Division investigators summarized their work for the advisory group at the **Annual Information Meeting.** Committee members visited individual laboratories and participated in informal discussions. An abbreviated program follows:

Opening Remarks	
R. F. Kimball	
Immunology	
Takashi Makinodan	Work of the Radiation Immunology group
Pathology and Physiology	
A. C. Upton	Work of Pathology-Physiology section
Mammalian Recovery	
C. C Congdon	Mortality from secondary disease
Developmental Biology	
Tuneo Yamada	Control of RNA synthesis in tissue transformation
Biochemistry	
Elliot Volkin	A biochemical approach to the problem of genetic recombination of bacteriophage
G. David Novelli	Survey of work on transfer RNA
M. P. Stulberg	Structure and function of phenylalanine tRNA
Molecular Anatomy	
N. G. Anderson	The MAN Program
Biophysics	
L. G. Caro	Chromosome replication in *Escherichia coli*
Genetics	
W. L. Russell	Mammalian radiation genetics
U. H. Ehling	Chemical mutagenesis in mammals
M. A Bender	Statistical karyotype analysis
F. J. de Serres	X-ray-induced inactivation and mutation-induction in Neurospora
Carcinogenesis	
Stanfield Rogers	Concerning the use of viruses to transmit specific genetic information
Paul Nettesheim	Lung changes in mice following chronic chemical inhalation exposure
Educational Activities	
R. C. Fuller	Current status of the graduate school
Concluding Remarks	
R. F. Kimball	

SYMPOSIUM ON BASIC MECHANISMS IN PHOTOCHEMISTRY AND PHOTOBIOLOGY

The seventh in the continuing series of Latin American conferences initiated in 1961 under the leadership of Alexander Hollaender, **The Symposium on Basic Mechanisms in Photochemistry and Photobiology,** was held in Caracas, Venezuela, December 4–8. The symposium was jointly arranged by the Instituto Venezolano de Investigaciones Científicas, the Universidad Central de Venezuela, the Biology Division of Oak Ridge National Laboratory, Brandeis University, and the U.S. National Academy of Sciences, with the support of the U.S. Atomic Energy Commission, National Science Foundation, Ford Foundation, and the Organization of American States.

The symposium's principal scientific goal was to focus on new knowledge acquired in recent years concerning the chemistry of nucleic acid and of heterocyclics, molecular spectroscopy, and photochemistry, toward an understanding of the physical and chemical bases of photobiological processes. Like

other meetings in this series, the conference served as an important educational experience for participants, including many advanced Latin American students, and not merely as a series of specialized discussions within isolated groups.

Serving on the symposium organizing committee were Alexander Hollaender, J. W. Longworth, R. O. Rahn, and R. B. Setlow of the Biology Division; Gabriel Chuchani and Marcel Roche, Instituto Venezolano de Investigaciones Científicas; and Henry Linschitz, Brandeis University.

The program was divided into the following sessions: Theoretical spectroscopy, Excited states reactions, Organic photochemistry, Photochemistry of purines and pyrimidines, Photoproducts in polynucleotides, and Experimental rates. Proceedings of the conference will be published in the *International Journal of Photochemistry and Photobiology*.

MEETING OF CANCER INSTITUTE DIRECTORS

The Biology Division was host for a meeting of the Association of Cancer Institute Directors December 11 and 12. Reports by Division investigators were presented on December 11 which covered activities in the Carcinogenesis and Molecular Anatomy programs.

1967 BIOMEDICAL PROGRAM DIRECTORS MEETINGS

Since February 1, 1967, Biology Division representatives have attended three meetings of the Biomedical Program Directors of the United States Atomic Energy Commission (USAEC). These meetings are held each year to review research in the various biomedical programs supported by USAEC's Division of Biology and Medicine at Commission laboratories.

R. F. Kimball and J. L. Liverman attended the winter meeting in New York City, February 13–14; S. F. Carson represented the Division at the spring meeting in Livermore, California, April 24–25; and Dr. Kimball attended the fall meeting at Brookhaven National Laboratory, Upton, Long Island, N.Y., October 9–10.

1967 BONE MARROW CONFERENCES

Since 1957 the Biology Division staff has cooperated with investigators from many institutions throughout the world in arranging bone marrow transplantation and chemical protection conferences **(Bone Marrow Conferences)**.

Abstracts of reports presented at each conference and special contributed papers are published in *Experimental Hematology*, a journal edited in the Biology Division with the assistance of the Technical Publications Department of the ORNL Technical Information Division. In 1967, three issues of *Experimental Hematology* (Nos. 12,[1] 13, and 14) were published, and a fourth issue (No. 15) is to be published by late February 1968. In addition, a special booklet which included collected programs from previous meetings was published in 1967.

In addition to writing a foreword and an explanation of the cover illustration that is selected for each issue, C. C Congdon coordinates material for the publications so that each issue is generally published within four months of the conference.

Biology Division members took an active part in three bone marrow meetings held during 1967:

[1] *Experimental Hematology* No. 12 primarily includes abstracts from the Bone Marrow Conference held December 2–3, 1966, at the University of Michigan, Ann Arbor.

April 17, at Chicago, Illinois

Bone Marrow Transplantation and Chemical Protection Meeting

Chairman: Jerry P. Lewis, Presbyterian–St. Luke's Hospital, Chicago.

Division Reports: The effect of hypoxia on mortality from secondary disease (C. C CONGDON,[2] M. L. Simmons, and R. E. Toya); Serine metabolism in radiation chimeras (A. L. KRETCHMAR[3]); Graft-vs-host reaction in man produced by thoracic duct (HELEN VODOPICK,[4] C. C Congdon, C. L. Edwards, N. Gengozian, B. Nelson, and G. A. Andrews).

Proceedings: Covered in *Experimental Hematology* No. 13.

June 26, at Paris, France

Conference on Bone Marrow Transplantation and the Physiology of Hemopoietic Tissues

Chairman: Leon Schwarzenberg, Institute de Cancerologie et d'Immunogenetique, Villejuif, France.

Division Report: Serine metabolism in radiation chimeras (A. L. KRETCHMAR[3]).

Proceedings: Covered in *Experimental Hematology* No. 14. This issue also includes two contributed papers (i.e., written especially for the journal) and a special section on "Laboratory Animal Hematology." Contributed papers were: (1) Carbon-14 activity in respiratory CO_2 of mice treated with methotrexate and injected with L-3-^{14}C-serine (A. L. Kretchmar and D. G. Doherty); and (2) Note on the reduced number of hemopoietic colonies in spleens of old mice (A. L. Kretchmar and W. R. Conover). Division contributions to the special section were: (1) Introduction (M. L. Simmons); (2) Observations on a leukemia-like disease in C57BL/6 mice (C. B. Richter and J. O. Brick); and (3) Incidence of murine virus antibody in humans in contact with experimental animals (R. W. Tennant, R. K. Reynolds, and K. R. Layman).

November 17–18, at Oak Ridge, Tennessee

10th Anniversary Bone Marrow Conference

Chairman: Alexander Hollaender, Biology Division, Oak Ridge National Laboratory

Division Reports: Ultrastructure of antigen localization and virus-like particles in splenic germinal centers (A. K. SZAKAL and M. G. Hanna, Jr.); The effect of estradiol on hemopoiesis in the RF mouse (V. K. JENKINS); Chemical protection against radiation damage: a review (D. G. DOHERTY); Enhanced infection with *Mycobacterium leprae* in thymectomized-irradiated mice (CHARLES C. SHEPARD[5] and C. C Congdon).

Proceedings: Covered in *Experimental Hematology* No. 15. This issue also includes one contributed paper (Kidney lysozyme in hypoxic radiation chimeras, by Eiji Endo) and a special section (abstracts from "Workshop on Immunology of Leprosy," a meeting held October 17, 1967, at Silver Spring, Maryland). An abstract by C. C Congdon entitled "Lymphatic tissue pathology in radiation chimeras" is included in the special section.

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[2]Names of scientists who made presentation are shown in capital letters throughout this section.

[3]Division Consultant.

[4]Dr. Vodopick and coauthors other than Dr. Congdon are associated with the Medical Division of the Oak Ridge Institute of Nuclear Studies, an operating unit of Oak Ridge Associated Universities.

[5]Dr. Shepard is associated with the National Communicable Diseases Center, Atlanta, Georgia.

1968

TENTATIVE PROGRAM: TWENTY-FIRST ANNUAL BIOLOGY RESEARCH CONFERENCE

The 1968 Research Conference on **Molecular Aspects of Differentiation**, sponsored by the Biology Division of the Oak Ridge National Laboratory, will be held April 8–11 at the Riverside Motor Lodge in Gatlinburg, Tennessee. The organizing committee – Tuneo Yamada (chairman), J. F. Albright, F. J. Finamore, F. T. Kenney, John Papaconstantinou, and R. A. Wallace – has announced the following tentative program:

Monday, April 8

Morning Session

Control of Protein Synthesis in Tissue Differentiation

Chairman – J. D. Ebert, Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland

Multiphasic Regulation of Specific Protein Synthesis in Cytodifferentiation – W. J. Rutter, Division of Biochemistry, University of Washington, Seattle, Washington

Muscle Differentiation and Macromolecular Synthesis – J. R. Coleman and Annette W. Coleman, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island

Afternoon Session

Synthesis of Specific Proteins in Induced Tissue Differentiation

Chairman – A. A. Moscona, Department of Zoology, University of Chicago, Chicago, Illinois

Chondrogenesis: Genotypic and Phenotypic Expression – J. W. Lash, Department of Anatomy, University of Pennsylvania, Philadelphia, Pennsylvania

Lens-Specific Antigens and Cytodifferentiation in the Developing Lens – Johan Zwaan, Department of Anatomy, School of Medicine, University of Virginia, Charlottesville, Virginia

Tuesday, April 9

Morning Session

Molecular Aspects of Oogenesis and Early Development

Chairman – Alberto Monroy, Istituto di Anatomia Comparata, Università di Palermo, Palermo, Italy

The Induced Synthesis and Transport of Yolk Proteins and Their Accumulation by the Oocyte – R. A. Wallace and J. N. Dumont, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Discussant – B. K. Follett, Leeds University, Leeds, Great Britain

The Pattern of Transcription and Translation in Echinoderm Development – P. R. Gross, Massachusetts Institute of Technology, Cambridge, Massachusetts

Factors Involved in Organelle Formation

Chairman – Folke Skoog, Department of Botany, University of Wisconsin, Madison, Wisconsin

The Developmental Aspect of Chloroplast Continuity in Euglena – J. A. Schiff and M. H. Zeldin, Department of Biology, Brandeis University, Waltham, Massachusetts

Afternoon – Free

Wednesday, April 10

Morning Session

Controlling Factors in Morphogenesis

Chairman – C. H. Waddington, Institute of Animal Genetics, Edinburgh, Scotland

Factors Determining Embryonic Differentiation – Heinz Tiedemann, Physiologisch-Chemisches Institut der Freien Universität, Berlin, Germany

Factors Controlling Morphogenesis in the Slime Mold – Barbara Wright, Institute of Biological and Medical Sciences, Retina Foundation, Boston, Massachusetts

Afternoon Session

Molecular Events in Terminal Differentiation

Chairman – Harold P. Rusch, McArdle Memorial Laboratory, The University of Wisconsin, the Medical School, Madison, Wis.

Regulation of Ribosomal RNA Synthesis and Assembly in Vertebrate Lens Cell Differentiation – John Papaconstantinou and Emilia M. Julku, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Messenger RNA in Avian Erythroblasts at the Levels of Transcription and Translation – Klaus Scherrer, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland

Thursday, April 11

Discussion Session

Leader – C. L. Markert, Department of Biology, Yale University, New Haven, Connecticut

Present Status and Perspectives in the Study of Cytodifferentiation at the Molecular Level

Panel:

P. R. Gross, Massachusetts Institute of Technology, Cambridge, Massachusetts

Donald Kimmel, Brown University, Providence, Rhode Island

D. L. Nanney, University of Illinois, Urbana, Illinois

W. J. Rutter, University of Washington, Seattle, Washington

INTERNATIONAL SYMPOSIUM ON NUCLEAR PHYSIOLOGY AND DIFFERENTIATION

Plans are well under way for the eighth in the series of Latin American conferences held annually since 1961.

This conference, on **Nuclear Physiology and Differentiation**, will be held at Belo Horizonte, Brazil, from December 1 through 6, 1968. The meeting will be cosponsored by the University of Texas, the Biology Division, and several Brazilian research and educational institutions.

Honorary president of the symposium committee is Carlos Chagas, Brazilian Permanent Delegate to UNESCO. Division members serving on the committee are: Alexander Hollaender, R. F. Kimball, O. L. Miller, Jr., Elliot Volkin, and R. C. von Borstel.

1968 BONE MARROW CONFERENCES

Tentative plans have been made for two 1968 **Bone Marrow Conferences** – the first to be held in Atlantic City, New Jersey, April 17, in conjunction with the Federation of American Societies for Experimental Biology meetings, and the second planned for the University of Padua, Italy, June 26–28.

The latter meeting (**II Conference on Germinal Centers of Lymphatic Tissue**) is to be similar to the international conference held in Bern, Switzerland, in June 1966. The main topics of the scientific program will be: Development and morphology of germinal centers, Cellular kinetics of lymphoreticular tissue with special reference to germinal centers, Germinal centers in relation to antigen localization and antibody production, Cellular immunity and germinal centers, and Human pathology and germinal centers. Dr. L. Fiore-Donati, Istituto di Anatomia Patologica, University of Padua, Padua, Italy, is organizing the program.

Educational Activities

STUDENT TRAINEE PROGRAM

Twenty juniors from colleges in 16 states spent the summer of 1967 in the Biology Division on temporary appointments. The Summer Student Trainee Program is administered for the United States Atomic Energy Commission by Oak Ridge Associated Universities and is carried out in cooperation with the Oak Ridge National Laboratory. Each student carried out a specific project under the supervision and guidance of a senior staff member and presented final oral and written reports on his summer's work.

The 1967 students, their affiliations, and the sections to which they were assigned were:

NAME	SCHOOL	GROUP
Nancy Lee Antonsen	Arkansas College Batesville, Arkansas	Mammalian Recovery
Deborah Elizabeth Charsha	University of Texas at El Paso Texas Western College El Paso, Texas	Pathology and Physiology
Martha Ellen Depinet	Siena Heights College Adrian, Michigan	Enzymology

NAME	SCHOOL	GROUP
Linda Emma Duguay	University of Rhode Island Kingston, Rhode Island	Pathology and Physiology
Robert I. Fox	University of Michigan Ann Arbor, Michigan	Biophysics
Steven Henikoff	University of Chicago Chicago, Illinois	Cell Physiology
Kathleen Jeannette LaBrot	University of Wisconsin Madison, Wisconsin	Yeast, Hymenoptera, and Phage Genetics
Faith Eloise Mace	West Virginia University Morgantown, West Virginia	Effects of Radiation on Paramecium
Teackle Wallis Martin, Jr.	Southwestern at Memphis Memphis, Tennessee	Enzymology
Kathryn Murray	Brigham Young University Provo, Utah	Yeast, Hymenoptera, and Phage Genetics
Charles Lloyd Paxson	Kansas State College Pittsburg, Kansas	Mammalian Recovery
Linda Lea Peterson	University of Wisconsin Madison, Wisconsin	Biophysics
John Aubrey Rock	Louisiana State University Baton Rouge, Louisiana	Rhynchosciara Genetics
Diana Marie Rudack	Wisconsin State University Eau Claire, Wisconsin	Cell Growth and Differentiation
Sally Sherard	Randolph-Macon Woman's College Lynchburg, Virginia	Nucleic Acid Enzymology
Paula Jane Thompson	University of Wisconsin Milwaukee, Wisconsin	Plant Physiology
Anne-Marie Tucker	Regis College Weston, Massachusetts	Biophysics
Glenda Carol Webb	Dillard University New Orleans, Louisiana	Radiation Immunology
Eric A. Wulfsberg	Luther College Decorah, Iowa	Pathology and Physiology
Francis James Zonay	Moravian College Bethlehem, Pennsylvania	Nucleic Acid Enzymology

OTHER STUDENTS

The following nine undergraduate students also worked in the Division on temporary summer appointments in 1967.

NAME	SCHOOL	GROUP
Michael Edwin Brannom	Auburn University Auburn, Alabama	Inhalation Carcinogenesis

NAME	SCHOOL	GROUP
William Hopkins Britt	Tennessee Technological University Cookeville, Tennessee	Experimental Animal Facility
Johnny Eugene Burris	King College Bristol, Tennessee	Experimental Animal Facility
Bonnie Kaye Ferrill	Tennessee Technological University Cookeville, Tennessee	Administrative Office
Susan Kay Haeusler	University of Tennessee Knoxville, Tennessee	Administrative Office
Clarence David Heidel, Jr.	University of Tennessee Knoxville, Tennessee	Engineering Office
Katherine Settles	Knoxville College Knoxville, Tennessee	Media Kitchen
Larry Burl Shipley	East Tennessee State University Johnson City, Tennessee	Mammalian Genetics Section
Carole Sue Turbyville	University of Tennessee Knoxville, Tennessee	Administrative Office

SENIOR TRAINEES

Seven students who had either been accepted at graduate schools for the fall of 1967, or were already enrolled in graduate school, were employed during the summer to carry out independent research projects in the Division. Listed below are their names, the graduate schools they are now attending, and the group with which they were associated during the summer:

NAME	SCHOOL	GROUP
Donald Craig Brater[1]	Duke University Durham, North Carolina	Molecular Anatomy
Richard Albert Dolbeer[1]	University of Tennessee Knoxville, Tennessee	Nucleic Acid Enzymology
Daniel Richard Holladay	Auburn University Auburn, Alabama	Enzymology
John Loren Lenz	Kansas State University Manhattan, Kansas	Experimental Animal Facility
Jane Ann Mays[1]	Vanderbilt University Nashville, Tennessee	Radiation Microbiology and Microbial Genetics
Paul Bruce Selby[1]	UT – Oak Ridge Graduate School of Biomedical Sciences Oak Ridge, Tennessee	Mammalian Genetics
Kent Westbrook Wilcox[1]	Johns Hopkins University Baltimore, Maryland	Molecular Anatomy

[1]Entered Graduate School, Fall 1967.

UT-OAK RIDGE GRADUATE SCHOOL OF BIOMEDICAL SCIENCES

Established in 1966 by the University of Tennessee Graduate School in cooperation with the Biology Division of the Oak Ridge National Laboratory, the UT-Oak Ridge Graduate School of Biomedical Sciences began its first classes in September 1967 with seven highly qualified students. Courses taught during the fall quarter were Biochemistry, Cell Biology, Transmission Genetics, and Doctoral Research and Dissertation, as well as a student seminar in the biomedical sciences and a laboratory rotation course. Additional courses will be offered in the winter and spring quarters.

The full-time Biomedical School faculty consists of the Director – R. C. Fuller, J. S. Cook, and D. E. Olins. Twenty-one members of the Biology Division have part-time staff appointments; these are listed below. Further appointments will be made as new courses and activities develop.

Thesis research is done in the Biology Division under the supervision of senior staff members, and research topics may be selected from a variety of areas, including: microbiology, cell physiology, immunology, biochemistry, biophysics, cytology, radiation biology, virology, developmental biology, carcinogenesis, plant physiology and photosynthesis, and experimental pathology.

Recruiting is now under way for future classes, and plans are being formulated for the construction of a research training building adjacent to the Biology Division. (The school is presently housed within the Division in space rented to the University.)

Part-Time Staff Appointments of Biology Division Personnel to Graduate School of Biomedical Sciences

H. I. Adler
N. G. Anderson
W. E. Barnett
S. F. Carson
Roy Curtiss III
D. G. Doherty
J. L. Epler
F. J. Finamore
E. H. Grell
Alexander Hollaender
Ann B. Jacobson
K. Bruce Jacobson
R. F. Kimball
J. L. Liverman
M. Ellen Mattingly
O. L. Miller
G. David Novelli
J. D. Regan
W. L. Russell
R. B. Setlow
C. M. Steinberg

GRADUATE COURSES TAUGHT

UT–ORNL–Ford Foundation Program. – During the past year, the Biology Division continued its active participation in the University of Tennessee–Oak Ridge National Laboratory Cooperative Research Program sponsored by the Ford Foundation. The following are Division members who took part in the program:

H. I. Adler taught a course in Bacterial Genetics for the University's Department of Microbiology during the winter quarter. This course dealt primarily with bacterial cytology, mutations, DNA as genetic material, and genetic systems of bacteria and bacterial viruses.

During the winter and spring quarters, C. C Congdon taught a course in Pathological Processes. It dealt with the concepts and properties of steady state interruptions in biological systems.

L. H. Smith – together with R. W. Holton, Head of the University's Department of Botany – taught a winter quarter course in Cell Physiology that concentrated on the functional activities of cells, with special emphasis on the physiochemical phenomena of protoplasm; organization in the cell; and the transport of materials, as well as the release, sources, and uses of energy, in the cell.

A. H. Haber taught a course entitled Developmental Plant Physiology during the spring quarter. This course dealt with intracellular and intercellular regulation, mathematical descriptions of growth and morphogenesis, hormonal regulation of growth and development, and relation of cell division to development; it included laboratory work.

UT-Oak Ridge Resident Graduate Program. – The Biology Division has, for many years, cooperated with the Oak Ridge Associated Universities (ORAU*) and the University of Tennessee by supplying instructors for their jointly sponsored Resident Graduate Program.† This past year has been no exception. The following are courses taught by Division members under this program:

Advanced Genetics – W. E. Barnett – places major emphasis on modern trends and theories in genetics.

Biometry – D. G. Gosslee – places emphasis on methods of planning biological experiments and methods of analysis and interpretation of the data.

Cellular and Comparative Biochemistry – F. J. Finamore – covers such topics as metabolism energy transformations, the properties of enzymes, and enzyme kinetics.

Concepts in General Zoology – G. L. Whitson – places emphasis on such problems as cell morphology, reproduction, genetics, and cell functions.

General Genetics – W. E. Barnett – is concerned with the fundamental principles of heredity.

Histology – W. D. Gude – involves primarily the study of all principal tissues, organs, and organ systems of the mammalian body, with emphasis on the recognition and identification of all tissues studied.

Human Genetics – J. D. Regan – covers human cytogenetics and chromosomal abnormalities, the metabolic lesions in man which are associated with hereditary disease, and the molecular diseases of man due to genetic abnormalities in structural and functional macromolecules.

LECTURES GIVEN IN OTHER COURSES, INSTITUTES, AND SPECIAL PROGRAMS[1]

J. F. Albright participated as a lecturer in the International Training Course in Immunology at the National Atomic Energy Commission in Buenos Aires, Argentina. He gave ten lectures and conducted five laboratory sessions from September 4 to 18. This course was offered to postgraduate students from various South American countries.

N. G. Anderson, W. E. Barnett, W. L. Carrier, D. G. Doherty, W. D. Fisher, J. G. Green, W. D. Gude, Warren Harris, D. J. Nelson, R. A. Popp, Stanfield Rogers, A. C. Upton, Mayo Uziel, and R. C. von Borstel have all given lectures in G. L. Whitson's course, Concepts in General Zoology.

N. G. Anderson, N. K. Clapp, C. C Congdon, J. S. Cook, E. B. Darden, Jr., D. G. Doherty, and W. D. Gude spoke at the 7th Annual U.S. Army Nuclear Science Seminar, held during July in Oak Ridge, Tennessee.

L. G. Caro was one of the lecturers in a three-week course on the Application of Electron Microscopy to Molecular Biology, organized by himself and Professor Edouard Kellenberger and sponsored by the European Molecular Biology Organization. This course was given at the Institute of Molecular Biology in Geneva, Switzerland, September 4–23, and was offered to students at the post- or advanced doctoral level who had a good background in some branch of molecular biology.

G. E. Cosgrove, in April, lectured on "Delayed effects of low-level radiation" in the ORAU Medical Radioisotopes Course, and in July and September gave the same talk in the ORAU Health Physics Summer Institute and the ORAU Health Physics Course, respectively.

Roy Curtiss III lectured on "Quantitative analysis in virology" and "Radiobiology of bacterial viruses" for the Department of Microbiology of the University of Tennessee in February. These lectures

*ORAU is an organization of 41 colleges and universities linked together in a common nuclear energy educational program; its administrative, training, and research facilities are located in Oak Ridge, Tennessee.

†All courses under this program are taught at the ORAU Special Training Building, Laboratory Road, Oak Ridge.

[1]For lecture titles not given in this section, see **Lectures**, p. 16.

were given as part of a course in Virology. In June, Dr. Curtiss participated in a Bacterial Genetics course held at the Cold Spring Harbor Laboratory of Quantitative Biology. His subject was "Mechanisms of chromosome mobilization and transfer during bacterial conjugation."

U. H. Ehling gave two lectures, "Radiation biology – genetic effects" and "Mammalian genetics," at the ORAU Summer Institute in Health Physics and the ORAU Summer Institute in Radiation Biology, respectively.

W. D. Gude lectured on "Autoradiography" during the winter quarter of the ORAU Medical Radioisotopes Course.

Alexander Hollaender, J. L. Liverman, Liane B. Russell, and W. L. Russell were speakers at the Conference on Science for Clergymen, sponsored by the National Science Foundation and the Alfred P. Sloan Foundation, held in Oak Ridge, Tennessee, during August.

F. T. Kenney participated December 12 as a lecturer in the Special Topics in Biochemistry lecture series given at the Medical School, University of Missouri, Columbia, Missouri. His subject was "Regulation of enzyme synthesis and degradation in mammalian tissues."

Takashi Makinodan gave a lecture on "Radiation immunology" at the University of Tennessee, May 18, as a participant in a Radiation Physiology course, and he lectured and directed laboratory sessions on "Cellular aspects of immune competence" in the International Training Course in Immunology held in Buenos Aires, Argentina, during the fall.

E. F. Oakberg lectured on "Radiation genetics" and "Radiation response of gonads" in a graduate course in Radiation Biology given at the University of California, Berkeley, during the spring quarter. He also lectured on "Radiation damage to the gonads" at the University of Tennessee, April 22, as part of a Radiation Physiology course.

C. T. Rankin, Jr., assisted in directing a course on Zonal and Continuous Flow Centrifugation in the Model L4 for the Spinco Division of Beckman Instruments, Inc., July 29–August 5.

W. L. Russell participated as a guest lecturer in a special course given at the Buffalo School of Medicine, State University of New York, Buffalo, New York, during the spring. On October 13, he spoke to a group of Health Physics students at the ORAU Special Training Building about "Genetic effects from radiation exposure."

Jane K. Setlow spoke on the "Photochemistry of DNA" at the ORAU-sponsored Radiation Biology Institute held during August in Oak Ridge, Tennessee.

C. M. Steinberg gave a series of lectures in a course on Bacterial Viruses at the Cold Spring Harbor Laboratory for Quantitative Biology, July 3–August 18.

P. A. Swenson participated as a guest lecturer in a Radiation Biology course given at the University of Tennessee during the spring quarter.

A. C. Upton lectured on the "Biological effects of low level irradiation" in the ORAU Medical Radioisotope Course given in Oak Ridge, Tennessee during the winter quarter.

R. C. von Borstel conducted a short course on Chromosome Structure and Function at the Atomic Energy Center in Lahore, Pakistan, in September.

SPECIAL COURSES TAKEN

J. O. Brick attended a short course on Radiographic Techniques and X-ray Equipment, given at the College of Veterinary Medicine, Ohio State University, March 22–23.

E. L. Candler, Jr., was in Tarrytown, New York, from March 6 to 10, for the Autoanalyzer Training Course sponsored by Technicon Controls, Inc.

Julia W. Deitchman took a short course on Thin-Layer Chromatography at the University of Tennessee on March 21 and 22. This course was sponsored by the American Chemical Society.

Ann B. Jacobson attended a course in Bacterial Viruses at the Cold Spring Harbor Laboratory of Quantitative Biology, Long Island, New York, July 5–August 4.

S. P. Leibo was a student in the three-week course on the Application of Electron Microscopy to Molecular Biology given at the Institute of Molecular Biology, Geneva, Switzerland, September 4–23.

D. E. Olins attended a course on Electron Microscopy given at the University of California, Berkeley, from June 16 to July 3.

C. B. Richter took a course on Electron Microscopy at the University of Pittsburgh, Pittsburgh, Pennsylvania. The course began on October 23 and lasted through the 27th.

Mary L. Hummel Welch was in Palo Alto, California, May 15–19, for the Beckman Amino Acid Analyzer Training Course.

International Activities

Alexander Hollaender

The first two and one-half months of 1967 were spent on a trip abroad visiting European laboratories and Thailand, Pakistan, and India in the Far East. Arrangements have been made with Pakistan for a Molecular Biology Course to be given by members of the Biology Division, as well as by others, beginning in the Fall of 1968, to be financed entirely by the Pakistan Atomic Energy Commission. Also, arrangements were made for a possible symposium at the conclusion of this course in 1969.

A considerable number of laboratories in northern India were visited and arrangements made for a Symposium on Fundamental Radiobiology to be held at the Bhabha Research Centre in February 1969. Also, a number of contacts were made and preparations begun for cooperation with Indian scientists.

In July, at the invitation of the Brazilian Academy, three members of the Biology Division (Russell, Upton, and myself) attended a meeting on Peaceful Aspects of Atomic Energy held in Rio de Janeiro. The papers of this symposium are being published by the Brazilian Academy in a special volume. During this visit an inspection trip was made to Belo Horizonte to prepare for the 1968 symposium.

In October of 1967 the Ford Foundation contributed a substantial amount of funds for two years for the support of these Latin American symposia. The money is to be used to support Latin Americans to attend these symposia, a modest amount of money is for the travel of the organizers for future symposia, and also some contribution is made toward the editing and publication of these symposia. This grant is being administered by the Latin American group in the Office of the Foreign Secretary at the U.S. National Academy of Sciences.

In November and December of this year I visited Sao Paulo and two cities in the Nordeste area of Brazil, Recife and Fortaleza, an important section of Brazil which has been neglected for many years and is now coming to the forefront in industrial and educational activities.

The Seventh International Latin American Symposium on Photochemistry and Photobiology was held in Caracas in December. It was a very successful meeting with many Latin Americans attending. The proceedings of this symposium are being prepared for publication. The proceedings of the 1966 Mexico City symposium on Enzymatic Aspects of Metabolic Regulation have been published as National Cancer Institute Monograph 27, November 1967.

We cooperated with the Puerto Rico University Biology Department in organizing a course in Modern Biology. The course is now being given at Rio Piedras, with Dr. Billen from the University of Florida in charge; and one professor from the University of Texas at Houston, one from Pennsylvania State University, and two scientists from the Biology Division, Oak Ridge National Laboratory each giving three weeks of lectures and laboratories.

Considerable effort was put into trying to convince pharmacologists, as well as other people, of the importance of chemical mutagenesis. It could very well become a kind of focal point for further development of cooperation with different government agencies in building up new approaches to basic biology

using the same type of approach which has been used in the radiation field. These projects are still under discussion.

Considerable effort was spent on developing the idea of setting up a long-term planning institute in biomedical and health sciences. A prospectus was prepared, several meetings were held, and the National Academy of Sciences is now considering possible sponsorship of this institute with the cooperation of a number of outstanding universities. (See also section **Foreign Travel.**)

Division Members Working in Other Laboratories

RETURNED FROM LABORATORIES ABROAD

J. G. Brewen returned from Australia in October 1967, having spent a year at the Commonwealth Scientific Industrial Research Organization in Canberra working with Dr. W. J. Peacock on radiation-induced chromosome aberration studies. Dr. Brewen's stay was jointly sponsored by a U.S. Public Health Service Fellowship and the Oak Ridge National Laboratory.

L. G. Caro returned to the Laboratory in early April after having spent eight months working with Professor William Hayes at the Microbial Genetics Unit, Medical Research Council, Hammersmith Hospital, London, England, on mechanisms of DNA transfers in bacterial conjugation and in transduction. Dr. Caro's stay was supported by a National Science Foundation senior Postdoctoral Fellowship until mid-February, and by the Biology Division until his return.

TO CONTINUE EDUCATION

V. K. Jenkins, who was on leave of absence to complete course work and research leading to the Ph.D. degree under a U.S. Public Health Service Fellowship, was awarded the Ph.D. degree in December by the University of Tennessee, Knoxville. He rejoined the staff of the Pathology and Physiology Section on November 1, 1967.

J. G. Farrelly, whose leave of absence began on September 15, 1966, is continuing to pursue his studies and research for the Ph.D. degree in biochemistry at the University of Tennessee. He will remain on leave until his work has been completed.

M. L. Davis was granted a leave of absence from the Pathology and Physiology Section to carry out studies and research for the Ph.D. degree in radiation biology at the University of Tennessee under a U.S. Public Health Service Predoctoral Fellowship. His thesis research is being done in the Division's Mammalian Recovery Section under the direction of C. C Congdon.

R. T. Jones was granted a leave of absence from the Pathology and Physiology Section to carry out his studies under a U.S. Public Health Service Predoctoral Fellowship to work on a Ph.D. degree in bacteriology at the University of Tennessee. His thesis research will be carried out in the Division's Radiation Microbiology and Microbial Genetics Section under the direction of Roy Curtiss III.

AT THE ATOMIC ENERGY COMMISSION

G. E. Stapleton is spending a second year with the Division of Biology and Medicine of the Atomic Energy Commission, Washington, D.C., while on leave of absence from the Biology Division.

COLLABORATIVE RESEARCH

S. P. Leibo spent three months, September–November, at the Institute of Molecular Biology, University of Geneva, Geneva, Switzerland, conducting collaborative research with Professor Edouard Kellenberger.

R. A. McGrath spent six months at the University of California, Lawrence Radiation Laboratory, Berkeley, California, to carry out collaborative research with Dr. Robert Haynes.

1. Cytology and Genetics

R. F. Kimball[a]

Effects of Radiation on Paramecium (1.1–1.7)

R. F. Kimball[a]
G. L. Whitson
E. G. Bailiff[a]
A. A. Francis
Stella W. Perdue
Faith E. Mance[b]
Betsy M. Sutherland[c]

Molecular Photobiology of DNA (1.8–1.15)

Jane K. Setlow
B. E. B. Moseley[d]
M. P. Gordon[e]
M. E. Boling
Dianne C. Brown
Alice A. Mattingly

Mammalian Cytology and Cell Genetics (1.16–1.21)

E. H. Y. Chu[a]
Karin E. Buckton[d]
E. Virginia Merriam[f]
Tatsuo Omura
Sandra L. Bell[g]
Patricia A. Brimer[a]
Nina S. Hammer[a]
Patricia L. Wortham[a]

Mammalian Biochemical Genetics (1.22–1.25)

R. A. Popp
K. M. Hilse[d]
W. S. McEwen
Diana M. Popp

Rhynchosciara Cytology (1.26–1.30)

Ellen Mattingly
Crodowaldo Pavan[g]
Shirley P. Ogle
C. L. Parker
A. L. P. Perondini[d]
Ronald Eckhardt[h]
John A. Rock[b]
L. C. G. Simoes[d]

Chromosome Chemistry (1.31)

D. E. Olins[a,g]
Ada L. Olins[g]
Patricia C. Looby

Participation in Cooperative Programs:

AEC-NCI Cocarcinogenesis (26.14–26.16)

[a]Dual assignments
[b]Student trainee
[c]Oak Ridge Associated Universities Predoctoral Fellow
[d]Visiting investigator from abroad
[e]Loanee
[f]USPHS Predoctoral Fellow
[g]Consultant
[h]NASA Predoctoral Fellow

1.1 PHOTOREACTIVATION AND MUTAGENESIS IN *PARAMECIUM*

R. F. Kimball E. G. Bailiff

Elsewhere in this report[1] evidence is given for monomerization of thymine dimers in *Paramecium* by photoreactivating light from a "black light" source. As a step toward tying this evidence together with evidence for photoreactivation of mutation in this organism, a series of six replicate experiments were performed with three doses of 2537-A radiation from a G.E. germicidal lamp. Each dose group was divided into five subgroups which received different doses of radiation from a G.E. black light filtered by a piece of plate

glass to remove shorter wavelengths. The dose rate for the black light was approximately 14,000 ergs mm^{-2} min^{-1}. There was a single control group receiving no dose of either radiation. Earlier studies had shown that no detectable mutation is produced by even higher doses of black light.

The results are plotted in Fig. 1.1.1. It is clear that photoreactivation occurs. There is some reason to suspect some changes in the form of the dose curves, but the number of dose points is inadequate to establish this beyond doubt. Mutation is given in "*M*" units, which is a modified arc sine transformation of the fraction surviving from mutation. Thus the dose curves should eventually approach an upper limit.

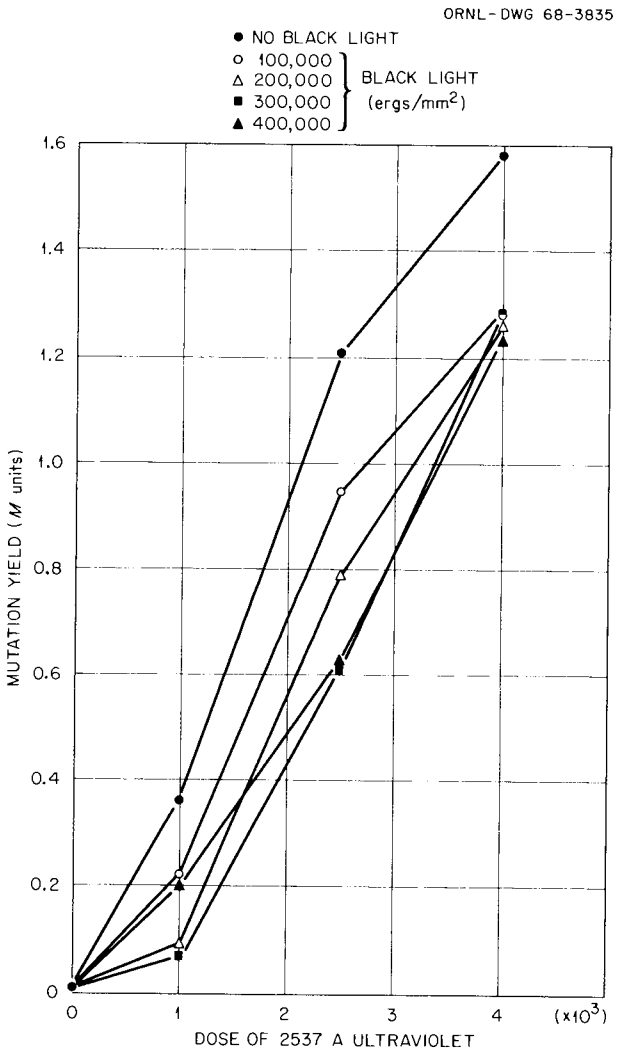

Fig. 1.1.1. Mutation Yield Plotted Against Dose of 2537-A Ultraviolet Radiation Followed by Different Doses of Black Light.

Figure 1.1.1 shows that a dose of approximately 300,000 ergs/mm^2 of black light appears to be sufficient for maximum reduction of mutation yield. At this dose, not all mutations are eliminated, and the dose-reduction factor is approximately 2 in the middle, approximately linear, portion of the dose curve. The simplest interpretation is that only about half the mutations come from pyrimidine dimers, since a dose of 300,000 ergs/mm^2 is sufficient to eliminate all dimers at the dose used in the chemical work.[1] Other interpretations cannot be excluded, however, including the possibility that maximum photoreactivation of dimers was not achieved by a dose that had the maximum effect on mutation.

References

[1]B. M. Sutherland, W. L. Carrier, and R. B. Setlow, this report, paper No. 25.15.

1.2 TESTS FOR TOXIC AND MUTATIONAL EFFECTS OF IRRADIATED SUCROSE IN *PARAMECIUM*

R. F. Kimball E. G. Bailiff

The possibility of mutagenic effects of irradiated sucrose was raised by reports of chromosome aberrations[1,2] produced by this material in mammalian cells. This is a problem of some concern for the practical application of radiation to food preservation. Attempts to produce recessive lethal mutations in *Paramecium* with 2% irradiated, autoclaved sucrose were unsuccessful. The irradiated, autoclaved sucrose was toxic to the paramecia, but subtoxic exposures produced no significant increase in the mutation rate. Chromosome aberrations should act as recessive lethals in the *Paramecium* system and thus should have been detected if they had occurred. The most probable explanation for the negative result is that the chromosome-breaking substance or substances in the irradiated, autoclaved sucrose does not penetrate to the nucleus of a cell as large as *Paramecium*. The alternative possibility – that mammalian chromosomes can be broken by the

substance, whereas *Paramecium* chromosomes cannot – cannot be excluded, however.

References

[1]M. W. Shaw and E. Hayes, *Nature* **211**, 1254 (1966).

[2]J. D. Regan, this report, paper No. 1.3.

1.3 CHROMOSOMAL DAMAGE INDUCED BY IRRADIATED, AUTOCLAVED SUCROSE SOLUTIONS

James D. Regan[1]

At the request of the Division of Biology and Medicine, some experiments were undertaken to provide further information on the reported[2] effect of irradiated sucrose solutions on human chromosomes.

Human lymphocyte cultures were prepared by the method of Moorhead *et al.*[2] Lymphocytes were obtained from human donors, treated with phytohemagglutinin to induce mitosis, and fixed for study 72 hr later. Sucrose solutions were added to a final concentration of 0.73%, a concentration shown by Shaw and Hayes[3] to be effective.

The following treatments were given: (1) not irradiated, sterilized through a Millipore filter without autoclaving (N.F.); (2) irradiated, filter sterilized (I.F.); (3) not irradiated, sterilized by autoclaving (N.A.); (4) irradiated, sterilized by autoclaving (I.A.). A pH indicator was present in the growth media and indicated that the pH remained between 7.2 and 7.4 in all cases.

The experiment was performed twice, and the results are shown as aberrations per 50 cells in Table 1.3.1. Fifty cells were scored per treatment except as noted. Our results confirm that irradiated, autoclaved sucrose produces large numbers of aberrations, as reported by Shaw and Hayes.[3] None of the other treatments produced significantly more aberrations than the N.F. control. The slight variations probably represent random fluctuations, not effects of the treatments. Thus neither irradiation nor autoclaving by itself under our conditions produced any detectable effect, and the results of both Shaw and Hayes[3] and ourselves must be attributed to one or more products resulting from the autoclaving of previously irradiated sucrose. Shaw and Hayes[3] sterilized only by autoclaving; they did not test the effects of irradiation alone.

References

[1]Human Cytogenetics section.

[2]P. Moorhead *et al.*, *Exptl. Cell Res.* **20**, 613 (1960).

[3]M. W. Shaw and E. Hayes, *Nature* **211**, 1254 (1966).

Table 1.3.1. Chromosomal Aberrations in Human Lymphocytes Treated with Irradiated Sucrose

Sucrose Solution	Aberrations per 50 Cells			
	Experiment 1		Experiment 2	
	Chromatid Aberrations	Achromatic Lesions	Chromatid Aberrations	Achromatic Lesions
N.F.	1	6	2	2
N.A.	2	10	2	2
I.F.	2	3	9	4
I.A.	24	26	11[a]	12[a]

[a]36 cells scored.

1.4 UV-INDUCED PYRIMIDINE DIMERS IN *TETRAHYMENA*. I. EXCISION

G. L. Whitson A. A. Francis
W. L. Carrier[1]

Evidence for repair synthesis of DNA in *Tetrahymena* during growth of cells in the dark after ultraviolet irradiation has been reported.[2,3] It was concluded from results of labeling experiments that incorporation of ^{32}P into DNA previously labeled with ^{3}H was into regions of parental DNA where excision of pyrimidine dimers had occurred. Our direct observations of the removal of pyrimidine dimers from cells in the dark after irradiation verify this inference.

Log-phase cells (*Tetrahymena pyriformis*, GL-C) grown in 1% proteose peptone with 0.1% liver extract and salts were labeled for several generations with ^{3}H-thymidine, then washed and resuspended in inorganic salt solution. Two-milliliter samples of cells (25,000 to 30,000 cells/ml) were irradiated in quartz cuvettes with a Hilger monochromator at 2652 A, 2520 ergs/mm^2. Aliquots of both irradiated and nonirradiated samples (taken immediately after uv and after growth in the dark for varying periods of time) were hydrolyzed with trifluoroacetic acid for 1 hr at 155°C. Pyrimidine dimers were isolated by chromatography with butanol-water in the first dimension and with saturated ammonium sulfate in the second dimension. The distribution of label was determined by standard scintillation counting methods on 1-cm strips using a dioxane-BBOT-naphthalene scintillator.

The results of a typical experiment are shown in Fig. 1.4.1. Pyrimidine dimers are lost from the trichloroacetic acid (TCA) insoluble fraction during postirradiation growth of cells in the dark. About 83% of these thymine-containing photoproducts disappear from cells after 47 hr. As expected, no photoproducts appeared in the control samples. While we observed no pyrimidine dimers in the TCA-soluble fraction immediately following uv (see Fig. 1.4.1), photoproducts accumulated in this fraction sometime during the first 8 hr of postirradiation growth of cells. It was apparent, however, that sometime later there was also a loss of pyrimidine dimers from this fraction. These results clearly indicate that (sometime after their excision from the DNA) photoproducts accumulating in the TCA-soluble fraction are lost from the cytoplasm of irradiated cells.

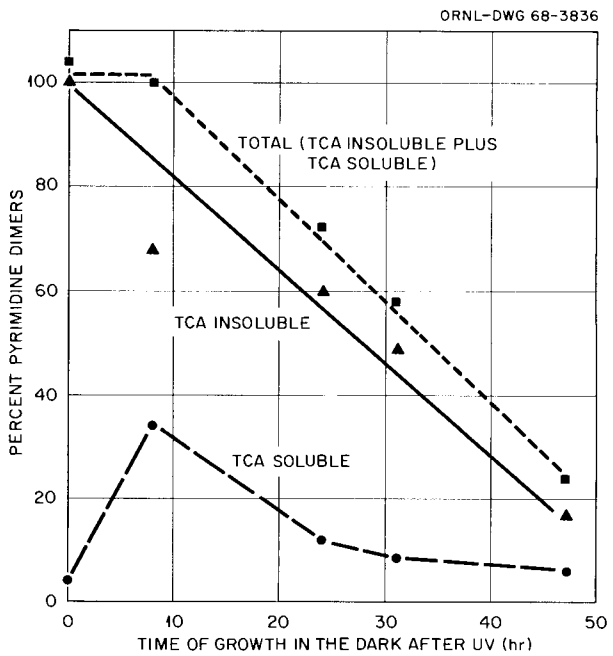

Fig. 1.4.1. The Percent Total Thymine-Containing Pyrimidine Dimers Observed in the TCA-Insoluble Fraction, TCA-Soluble Fraction, and Both Fractions Combined After Postirradiation Growth of Cells in the Dark at Varying Periods of Time. Uv = 2520 ergs/mm^2 at 2652 A.

References

[1] Biophysics section.
[2] C. F. Brunk and P. C. Hanawalt, *Abstracts 11th Ann. Meeting Biophys. Soc.*, p. 82 (1967).
[3] C. F. Brunk and P. C. Hanawalt, *Science* **158**, 663 (1967).

1.5 UV-INDUCED PYRIMIDINE DIMERS IN *TETRAHYMENA*. II. PHOTOREACTIVATION

G. L. Whitson A. A. Francis

It has already been inferred from studies on dark repair in *Tetrahymena* that exposure to white light cleaves pyrimidine dimers.[1,2] We have obtained direct results which indicate that long exposures to either white light, black light, or near uv (3650 A) destroy thymine-containing pyrimidine dimers in situ.

The techniques used in this study were essentially the same as presented in I above, except in this case controls consisted of both nonirradiated and irradiated samples (hydrolyzed immediately following uv treatment and hydrolyzed after growth in the dark for the same length of time as samples which were administered photoreactivation). Photoreactivation consisted of exposure to white light (two 15-w G.E. daylight fluorescent bulbs kept 4.5 cm from thin-layer samples using a ½-in. glass filter), black light (3000 to 4000 A, same conditions as above), or monochromatic light (3650 A).

The results of four separate experiments are shown in Fig. 1.5.1. Note that there was a loss in total percent radioactivity in dimer regions of chromatograms after long exposures to the different kinds of photoreactivating light. Controls are not shown, but they did not show comparable decreases (the irradiated controls) during the time of photoreactivation. The main difference in total percent dimers immediately following uv treatment was due to the difference in dose administered. Other differences were probably due to size differences in cells, accounting for difference in absorbed dose as well as light scattering. It was apparent, however, in all cases that pyrimidine dimers were monomerized by the various photoreactivating treatments. It appears that the time required for photoreactivation (complete monomerization) is largely dependent on the number of dimers initially present following uv irradiation. We previously reported biological reversal (photoreactivation) with short exposures to near uv light which involved only the disappearance of a few dimers.[3,4] It remains, therefore, to be seen whether or not there is a quantitative correlation between biological reversal and the monomerization of pyrimidine dimers.

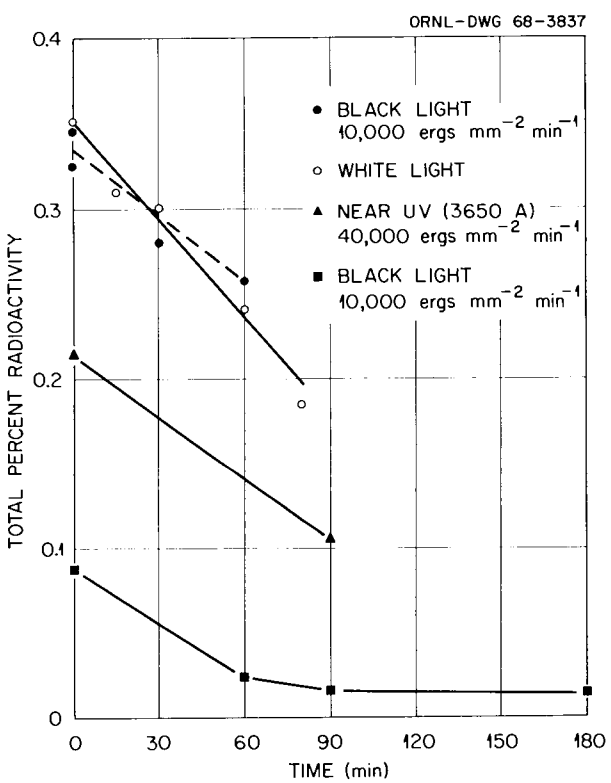

Fig. 1.5.1. The Loss in Total Percent Radioactivity in the Thymine-Containing Pyrimidine Dimer Regions of Chromatograms of UV-Irradiated *Tetrahymena* After Different Treatments of Photoreactivation.

References

[1]C. F. Brunk and P. C. Hanawalt, *Abstracts 11th Ann. Meeting Biophys. Soc.*, p. 82 (1967).

[2]C. F. Brunk and P. C. Hanawalt, *Science* **158**, 663 (1967).

[3]G. L. Whitson, A. A. Francis, and W. L. Carrier, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967*, ORNL-4100.

[4]G. L. Whitson, A. A. Francis, and W. L. Carrier, *J. Protozool.* **14** (suppl.), 8 (1967).

1.6 UV ACTION SPECTRUM OF SURVIVAL AND FORMATION OF THYMINE-CONTAINING PYRIMIDINE DIMERS IN *TETRAHYMENA*

A. A. Francis G. L. Whitson

Previous studies have shown that photoreactivation of uv-induced division delays can occur while less than 10% of the thymine-containing pyrimidine dimers are destroyed.[1] Although this finding does not eliminate pyrimidine dimers as the primary uv-induced lesion, it does make further comparisons of survival and dimer formation and repair after uv irradiation more important. This study is a comparison of the action spectrum of survival and pyrimidine dimer formation in *Tetrahymena*.

Cells grown in 1.0% proteose peptone with 0.1% liver extract and salts were washed and resuspended in inorganic salt medium prior to uv irra-

diation. Cells used for pyrimidine dimer determinations were grown overnight in the presence of ^{3}H-thymidine (10 μc/ml) and resuspended in unlabeled media for 2 hr prior to irradiation. ^{3}H-Thymidine-containing pyrimidine dimers were isolated by paper chromatography.[2] DNA was isolated by the method of Marmur.[3]

The action spectrum of survival determined by following the colonizing ability of single-cell isolates has a minimum at λ 2800 A, this wavelength being twice as effective in killing the cells as λ 2650 A (Fig. 1.6.1). The uv induction of pyrimidine dimers in vivo in *Tetrahymena* DNA has been found to have no such wavelength dependence, the same number of dimers forming at λ 2650 A and λ 2800 A with the same initial dose. In contrast to the in vivo finding, the formation of pyrimidine dimers in a solution of DNA isolated from *Tetrahymena* has been found to show strong wavelength dependence, with about twice the formation of dimers at λ 2650 A as at λ 2800 A. The differences in the action spectrum of dimer formation in vivo and in vitro are probably due to cytoplasmic shielding of the DNA in the cells. These results indicate that at λ 2800 A there is at least one unknown uv-induced lesion in addition to pyrimidine dimers in DNA which becomes lethal in *Tetrahymena*.

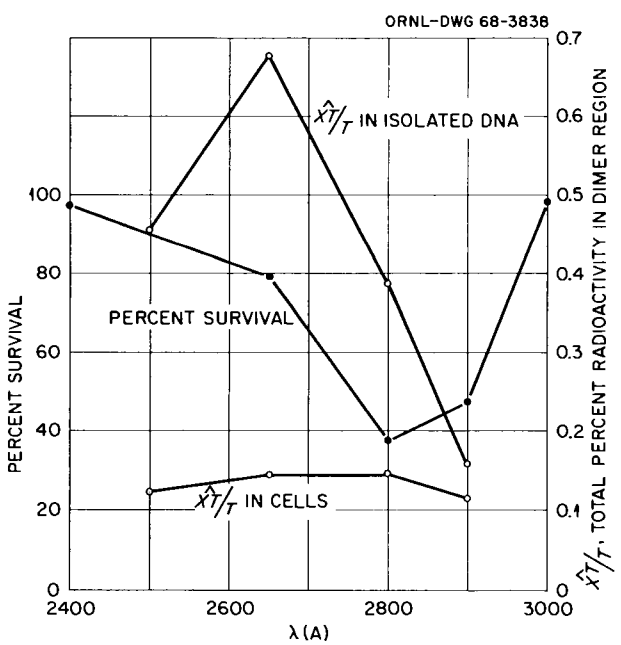

Fig. 1.6.1. The Effects of Different Wavelengths of UV Light on Survival of Cells and Formation of Thymine-Containing Pyrimidine Dimers in vivo and in vitro. The dose for the survival curve was 3750 ergs/mm^2; the dose for both dimer curves was 1000 ergs/mm^2.

References

[1] G. L. Whitson *et al.*, *J. Cell Biol.* **35**, 142A (1967).

[2] A. Wacker, H. Dellweg, and D. Weinblum, *Naturwissenschaften* **47**, 477 (1960).

[3] J. Marmur, *J. Mol. Biol.* **3**, 208 (1961).

1.7 THE ISOLATION OF DNA AND MITOCHONDRIA FROM *TETRAHYMENA*

G. L. Whitson A. A. Francis
W. D. Fisher[1]

It has been shown that DNA from *Tetrahymena* has a high A+T content (75%),[2] and CsCl centrifugation studies of DNA from whole cells showed two peaks of DNA, one with a buoyant density of $p = 1.688$ and the other (a smaller peak) with a density of $p = 1.680$.[3] Isolation of mitochondrial DNA from *T. pyriformis*, strain GL, indicated that it had a buoyant density of $p = 1.684$, and partial characterization of this DNA indicated that it was probably double-stranded native DNA.[4] We have also isolated whole-cell DNA from *T. pyriformis*, GL-C, and preliminary observations indicate that there are two peaks of DNA, one large peak with a buoyant density of about $p = 1.680$ and a smaller peak of about $p = 1.672$. We have also isolated mitochondria by sucrose gradient zonal centrifugation, but as of yet we have not obtained a large enough quantity for DNA isolation and density determinations. Our continuing interest in this problem is based on the following points: (1) Previous studies of buoyant densities of different DNA's show that DNA's with high A+T contents have low buoyant densities (poly d-AT, $p = 1.679$),[5] indicating the possibility that mitochondrial DNA from *Tetrahymena* may be of this type. (2) The base composition of mitochondrial DNA as well as the structural configuration of this DNA in *Tetrahymena* is unknown. Further investigations are planned to provide this information.

References

[1]General Physiology section.
[2]N. Sueoka, *Proc. Natl. Acad. Sci.* **47**, 1141 (1961).
[3]Y. Suyama and J. R. Preer, Jr., *Genetics* **52**, 1051 (1965).
[4]Y. Suyama, *Biochemistry* **5**, 2214 (1966).
[5]C. J. Schildkraut, J. Marmur, and P. Doty, *J. Mol. Biol.* **4**, 430 (1962).

1.8 ISOLATION AND CHARACTERIZATION OF UV-SENSITIVE MUTANTS OF *HEMOPHILUS INFLUENZAE*

Jane K. Setlow M. E. Boling
Dianne C. Brown Alice A. Mattingly

The isolation of radiation-sensitive mutants has been an important first step in the investigation of cellular repair mechanisms. *Hemophilus influenzae* has been selected for such a study because it has both a transformation system and a bacteriophage, so that it is possible to examine the cell's repair of transforming DNA and phage DNA as well as the repair of the cellular DNA.

H. influenzae cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and then selected for sensitivity to uv inactivation of colony formation by replica plating and spotting techniques. Seven mutants were isolated, following screening of about 30,000 individual colonies isolated from the replica plating screening procedure. The uv sensitivity of these mutants varies from 3 to 30 times that of the wild type. The following additional characterizations of the mutants and the wild type were made: (1) sensitivity to inactivation of colony-forming ability by x rays, (2) sensitivity to uv-induced filament formation, (3) ability to act as hosts to uv-irradiated *H. influenzae* phage, and (4) sensitivity of three markers on uv-irradiated transforming DNA with six of the mutants as recipients (the other mutant could not be transformed).

The seven mutants differ from the wild type and from each other by at least two of the sensitivity criteria. Only one of the mutants showed x-ray sensitivity greater than that of the wild type. All eight strains elongate following uv irradiation (up to a factor of about 7 increase in length), but the kinetics and the amount of elongation vary among the strains. The uv survival curves of the phage indicate that three of the mutants have reduced ability to repair phage DNA. The mutants show varying degrees of decreased ability to be transformed by uv-irradiated transforming DNA.

The three most sensitive mutants with respect to uv inactivation of colony formation are the same three which apparently are defective in repair of uv damage to phage. Therefore it is very probable that the same repair system (defective in these mutants) is used for phage and cellular DNA damage. There is some, but not complete, correlation of uv sensitivity of the mutants and their decreased ability to act as recipients for uv-irradiated transforming DNA, indicating that transforming DNA is repaired in the wild-type cell, again with the same repair system, but at least one other factor influencing the survival of transforming DNA must be introduced to explain the incomplete correlation. The fact that one of the uv-sensitive mutants is also x-ray sensitive suggests that there is at least one common step in the repair of uv and x-ray damage in *H. influenzae*.

1.9 THE MECHANISM OF REPAIR OF UV DAMAGE IN *HEMOPHILUS INFLUENZAE*

M. E. Boling M. P. Gordon
S. P. Modak[1] Alice A. Mattingly
Jane K. Setlow

In *Escherichia coli* the sensitivity of different strains to uv killing and the recovery of DNA synthesis following uv have been correlated with the ability of the cells to excise uv-induced pyrimidine dimers from the DNA.[2] In an attempt to understand the reasons for the increased uv sensitivities of the seven *Hemophilus influenzae* mutants, three types of measurements have been made on the mutants and the wild type: (1) DNA synthesis after very low uv doses; (2) pyrimidine dimers in DNA, in the acid-soluble fraction of the cells, and in the external medium at various times after uv; (3) the molecular weight of single strands of *H. influenzae* DNA at various times after uv, for the purpose of determining the mending of single-strand breaks introduced by the excision of dimers.

DNA synthesis after uv, measured as uptake of tritiated thymidine into acid-insoluble material, recovered in all eight strains. The recovery time, linearly related to dose, was very different among the strains and was shortest in the wild type. The

delay was largest in all strains when the cells were irradiated in early logarithmic growth, and smallest in early stationary phase.

The distribution of pyrimidine dimers after uv was measured by counting the radioactivity in chromatographically separated dimers in the external medium, and in the acid-insoluble and soluble fractions of cells prelabeled with tritiated thymidine. In the wild type and in four of the mutants, the dimers disappear from the acid-insoluble fraction of the cells after uv and appear in the external medium and, to a lesser extent, in the soluble cell fraction. The remaining three mutants apparently cannot excise dimers from their DNA.

Determination of the single-strand breaks made by excision involved lysing a mixture of unirradiated cells containing ^{14}C-labeled DNA and ^{3}H-DNA irradiated cells (previously allowed to incubate for various times) on an alkaline sucrose gradient. The lysed cells were centrifuged, and the molecular weights of the unirradiated and irradiated DNA calculated from the sedimentation velocities, as judged by the distribution of acid-insoluble radioactivity in drops collected from the centrifuge tubes. The single-strand molecular weight of the DNA of unirradiated or unincubated irradiated cells was one-fourth of the known molecular weight of *H. influenzae* DNA, which means that there is around one break per strand, either occurring naturally or as a result of the particular procedure we used. In all the strains except one, the molecular weight decreased with time of incubation following uv. In the wild type and in the excising mutants, the number of single-strand breaks was very small in comparison with the number of breaks which must have been made by the excision process, measured under the same conditions. In two of the nonexcising mutants the number of single-strand breaks was greater than in the case of the excising mutants. In the third nonexcising mutant no breaks appeared.

(1) Pyrimidine dimers are blocks to DNA synthesis in all the strains. The fact that recovery from the blocks also occurs in mutants which are unable to excise dimers indicates that these cells are able eventually to circumvent the blocks. The amount of uv-induced delay in DNA synthesis is only partly correlated with the presence or absence of excision among the strains. (2) The three mutants which lack an excision mechanism are the most uv-sensitive and also the least able to support the growth of uv-irradiated phage. Thus the excision mechanism must be involved in the repair of cellular and phage lethal damage. (3) The kinetics of mending of single-strand breaks by excising strains do not appear to differ appreciably. Therefore it will be necessary to look for other phenomena to account for the different uv sensitivities of these strains. (4) The fact that no single-strand breaks appear in the DNA of one of the nonexcising mutants following uv indicates that this mutant is unable to make the first cut next to the dimer. The other two nonexcising mutants, however, apparently can make this first break, but since the dimers are not excised, they are presumably unable to make the second break on the other side of the dimer.

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1.10 THE EFFECT OF A DNA REPAIR MECHANISM IN *HEMOPHILUS INFLUENZAE* ON THE PHOTOREACTIVABLE SECTOR OF TRANSFORMING DNA

M. P. Gordon Jane K. Setlow

The survival of uv-irradiated transforming DNA is greatly increased if the DNA is incubated in the light with photoreactivating enzyme from yeast before the DNA is assayed for its ability to transform cells. The photoreactivating enzyme is known to exert its biological effect by monomerizing uv-induced pyrimidine dimers. The photoreactivable (PR) sector is the fraction of the damage which is photoreactivable, or the fraction of dimer damage. A comparison of the PR sectors measured when the DNA is assayed on wild-type *Hemophilus influenzae* and on a uv-sensitive mutant whose repair system is defective should provide information on the relative repair of dimer damage and nonphotoreactivable damage in the wild-type cell.

The survival of transforming DNA irradiated with many different uv doses was measured with and without maximum photoreactivation with yeast enzyme, on wild type and on strain DB112, the most sensitive mutant, which lacks an excision repair mechanism. Because of the shape of the survival curves, which obey the relation $\sqrt{N_0/N} = 1 + CD$, where N_0 is the number of transformations with unirradiated DNA and N the number obtained

after a dose D, the sensitivity parameter (the constant C) may be obtained with good precision. The ratio C_{PR}/C was 0.05 in DB112 and 0.10 in the wild type. The PR sector is $1 - C_{PR}/C$, or 0.95 in DB112 and 0.90 in the wild type.

The PR sector is the pyrimidine dimer damage *not* repaired in the cell divided by the total damage not repaired in the cell. If the cell repairs dimers to a greater extent than it repairs nondimer damage, the PR sector will be lower than if there is little or no repair of either type of damage in the cell. Thus the lower PR sector obtained with the wild type suggests that dimer damage is repaired in the cell more efficiently than nondimer damage.

1.11 THE MECHANISM OF REPAIR OF X-RAY DAMAGE IN *HEMOPHILUS INFLUENZAE*

Gerald Price[1] Jane K. Setlow

One of the uv-sensitive *Hemophilus influenzae* mutants is more sensitive to x rays than the wild type and the six other uv-sensitive mutants. Investigation has been made of the ability of the wild type and the x-ray-sensitive strain to rejoin x-ray-induced single-strand breaks in an effort to determine why the two strains differ in sensitivity to ionizing radiation.

Unirradiated cells containing ^{14}C-labeled DNA were mixed with irradiated cells containing ^{3}H-labeled DNA and lysed on an alkaline sucrose gradient. From the relative sedimentation velocities of the acid-insoluble radioactive materials in the same gradient, the number of x-ray-induced single-strand breaks in the DNA of the irradiated cells may be calculated. An exposure of 20 kr produces about ten such breaks in either wild type or the mutant. When the wild type is incubated in nutrient medium following irradiation, the number of breaks decreases and becomes zero by 2 hr. The DNA of the sensitive mutant mostly breaks down into acid-soluble pieces during postirradiation incubation.

It is possible to account for the difference in x-ray sensitivity of the two strains in terms of their relative ability to rejoin x-ray-induced breaks in the DNA.

Reference

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1.12 DEFECTS IN TRANSFORMATION IN *HEMOPHILUS INFLUENZAE*

Gerald Price[1] Jane K. Setlow

Two of the uv-sensitive mutants of *Hemophilus influenzae* as originally isolated were unable to be transformed by *H. influenzae* transforming DNA. It was hoped that investigation of these two mutants would yield some information on the mechanism of transformation. Transformation could be blocked at the stage of entrance of the DNA into the cell or at the stage of integration of the DNA into the recipient cell genome.

One of the nontransforming mutants, DB113, was different from all the other strains in that subcultures from the original culture formed long filaments in logarithmic growth. Such subcultures could not be transformed. However, further subculture of this mutant caused a progressive decrease in the length of the filaments and at the same time a progressive increase in the ability to be transformed.

The second mutant, DB117, remained essentially nontransformable (with a transformation frequency 10^{-4} of that of the wild type) even after a series of subcultures. To determine whether transformation in this mutant is blocked at the cell-entrance step, measurement was made of the ability of the mutant and the wild-type cells to take up tritium-labeled DNA. The two strains took up the DNA to the same extent when they were grown to near saturation, but neither strain took up DNA during logarithmic growth.

Lack of transformation in DB113 is correlated with filament formation and thus probably involves a defect in the cell wall which prevents entrance of the transforming DNA. Lack of transformation in DB117, on the other hand, is probably a defect in the integration step. This strain has two other characteristics which may be relevant to this defect: (1) it fails to rejoin x-ray-induced single-strand breaks and (2) it has the longest uv-induced delay in DNA synthesis of all the mutants. Thus DB117 may be defective in transformation because of its inability to join one part of the transforming DNA to another single-stranded part of its own genome.

Reference

[1]Student, University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences.

1.13 THE EFFECT OF ACRIFLAVINE ON THE SENSITIVITY OF UV-IRRADIATED TRANSFORMING DNA

Jane K. Setlow M. E. Boling

Acriflavine mixed with uv-irradiated *Hemophilus influenzae* transforming DNA and competent cells appears to increase the uv sensitivity of the DNA.[1] It has been postulated by Patrick and Rupert[1] that this acriflavine effect is the result of inhibition of the cellular repair system. Since acriflavine affects the uv-resistant markers more than the sensitive markers in transforming DNA, it has been further postulated that resistant markers are resistant because they are repaired more efficiently in the cell.[1] A test of these hypotheses is possible with the use of uv-sensitive mutants of *H. influenzae* in which there is defective repair of transforming DNA by the cells.

The recipient cells were of six different types: wild type and five uv-sensitive mutants. Acriflavine has the smallest effect on transforming DNA sensitivity in the most sensitive mutant and even appears to *decrease* the sensitivity slightly. However, acriflavine increases the DNA sensitivity more in some strains with defective repair mechanisms than it does in the wild type. The ratio of the acriflavine effect on a resistant marker relative to that on a sensitive marker is relatively constant among the strains. The ratio of the sensitivities of the sensitive and resistant markers without acriflavine is in one case greater but usually is smaller than that of the wild type.

The Patrick and Rupert hypothesis would predict: (1) acriflavine would have the smallest effect in mutants with the most defective repair systems; (2) the effect should be largest in wild type; (3) the ratio of the acriflavine effect on the resistant relative to the sensitive marker should decrease markedly in mutants with defective repair systems; and (4) the ratio of the sensitivities of the sensitive and resistant markers should be lower in mutants with defective repair. Only parts of the first and fourth predictions are substantiated by our data. It is concluded that acriflavine does indeed interfere with repair of transforming DNA, but the dye must increase the sensitivity of transforming DNA by another mechanism as well, probably by decreasing the probability of recombination between irradiated DNA and recipient cells. The hypothesis that the difference in resistance of markers assayed in wild-type cells reflects the difference in repair is not substantiated by our data. It is concluded that different markers have different sensitivities, probably because of the variation in the probability of crossover per unit length of DNA around the markers, a probability which would be expected to be altered by acriflavine intercalating in the DNA.

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1.14 INTEGRATION OF PYRIMIDINE DIMERS IN TRANSFORMING DNA INTO THE RECIPIENT *HEMOPHILUS INFLUENZAE* CELL GENOME

Alice A. Mattingly Jane K. Setlow

UV-induced pyrimidine dimers have been shown to be responsible for most of the biological effects of uv inactivation of transforming DNA. It has also been shown that repair of uv-irradiated transforming DNA takes place in the recipient *Hemophilus influenzae* cell.[1] In order to know whether their repair may take place before or after integration of the transforming DNA into the genome of the recipient, it is important to determine whether or not pyrimidine dimers in the transforming DNA become integrated into the cell genome during transformation.

The criterion for integration was the ability of a lysate of competent cells which have been exposed to uv-irradiated transforming DNA to transform other competent cells with two genetic markers, one of which came from the original irradiated DNA and the other from the original competent cells. These two markers become linked upon integration, so that the frequency of double transformations is orders of magnitude higher than that expected on the basis of random interaction of the two markers in the second set of competent cells, which is the product of the frequencies of the single transformations.

The criterion for the presence of pyrimidine dimers in the integrated material was the increase in the ability of the lysate to cause the double transformations following photoreactivation of the lysate with photoreactivating enzyme from yeast.

The reactivating effect of this enzyme on uv-irradiated transforming DNA is due to the monomerization of pyrimidine dimers in the DNA. The frequency of double transformations from the lysates was appreciably higher after photoreactivation.

Some but not all the pyrimidine dimers in uv-irradiated transforming DNA become incorporated into the recipient genome. Thus it is probable that repair of irradiated transforming DNA can take place after integration.

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1.15 THE REPAIR OF UV DAMAGE IN WILD TYPE AND RADIATION-SENSITIVE MUTANTS OF *MICROCOCCUS RADIODURANS*

B. E. B. Moseley

Wild-type *M. radiodurans* is extremely resistant to uv radiation. Following irradiation it excises pyrimidine dimers from its DNA with such efficiency that the eventual death of the bacteria appears to be due equally to protein and nucleic acid damage, since the cells are equally sensitive to 280 and 265 nm radiation.[1] Two mutants of *M. radiodurans* have been isolated which are both about seven times more sensitive to uv than the wild type.[2] Some properties of the mutants which are relevant to their uv sensitivity have been studied.

UV survival curves of the mutants were obtained at wavelengths 280 and 265 nm. The mutants were more sensitive to 265 than to 280 nm radiation. The delay in DNA synthesis following sublethal doses of radiation was measured by incubating the irradiated cells in nutrient medium containing ^{3}H-thymidine. The wild type required about seven times the uv dose at 265 nm to cause the same time delay in DNA synthesis. The rate of excision of uv-induced pyrimidine dimers from the DNA was slightly smaller in the mutants. The repair of single-strand breaks in DNA caused by the excision of dimers was investigated. Cells with radioactively labeled DNA were irradiated with a sublethal dose of 3000 ergs/mm^2 at 265 nm and incubated for varying periods of time and then lysed, and their contents were centrifuged in alkaline sucrose gradients. The sedimentation velocity of the single-stranded DNA of the wild type indicated that after 15 min there were only two breaks in the DNA, although the excision data showed that 950 breaks must have been made by the excision process. In one of the mutants, following a similar but lethal radiation dose, the initial DNA damage was the same. However, after 15 min incubation, which results in the excision of somewhat fewer dimers than in the wild type, there were about ten breaks in the DNA.

The fact that the mutants are more sensitive to radiation at 265 than at 280 nm suggests that, unlike the wild type, all the lethal damage is to nucleic acid. The wild type excises pyrimidine dimers very efficiently, and the number of breaks in the DNA caused by excision which are open at any one time is a very small fraction of the total number of dimers excised ($<0.2\%$). The mutants are able to excise pyrimidine dimers from their DNA, but mend the breaks considerably less efficiently. This probably accounts for their uv sensitivity.

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1.16 ON THE REJOINING OF CHROMOSOME BREAKS INDUCED BY FRACTIONATED X-RAY EXPOSURES

E. H. Y. Chu Patricia C. Looby[1]

The classical theory of the formation of chromosome aberrations by radiation is that the radiation breaks the chromosomes and that these initial breaks rejoin. By using various doses and dose rates, Sax[2] concluded that radiation breaks chromosome threads immediately after the absorption of energy in the chromosomes and that these broken ends remained open or available for rejoining with other broken ends for only a limited time period. Lane[3] in *Tradescantia* microspores and more recently Evans[4] in human leukocytes in culture have obtained results which seemed at variance with Sax's conclusions. Both workers

found that the aberration yield fell to a minimum when the time between dose fractions was 2 to 4 hr. With increasing time intervals between fractions, however, the aberration yield increased until about 6 or 8 hr, when the yield obtained with a split dose was almost as high as that obtained with the full unsplit dose. A new hypothesis of chromosome aberrations as a consequence of misrepair was proposed.[4]

We decided to reexamine the situation by studying this possible Lane phenomenon in two in vitro mammalian cell systems: the human leukocytes and the mitotically synchronized Chinese hamster cells. The work on the latter is summarized here; results from the former can be seen in another report.[5]

An almost pure population of hamster cells in metaphase was obtained through combined treatments of colcemid and mechanical agitation. The cells reattached to glass surface and uniformly entered G_1 phase within 1 hr after inoculation. The particular cell line used (CH461901) has a long G_1 period to make this study possible. Preliminary experiments established the dose-effect relations of various aberrations, all of the chromosome type, induced at G_1. There was no apparent difference in radiosensitivity during this period of the cell cycle. A total dose of 200 r x rays was given either in one exposure or in two exposures separated by an interval ranging from 1 to 8 hr. The only aberrations scored were chromosome rings and dicentrics, which are easily identifiable and unmistakable. The 8-hr interval was the longest that could be tested in these cells, since chromatid-type aberrations induced either at late G_1 or early S began to appear in this series. Our data demonstrated clearly that within 2 hr the chromosome breaks induced by the first exposure no longer interacted with those induced by the second exposure and that there was no evidence of the Lane effect under the experimental conditions.

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1.17 IRRADIATION OF HUMAN PERIPHERAL BLOOD IN VITRO

Karin E. Buckton E. H. Y. Chu

A number of investigators have obtained dose relations in the induction of chromosome aberrations in human peripheral leukocytes x irradiated in vitro. While the experimental system offers one of the best opportunities for an evaluation of radiation hazards in man,[1] there remain a number of technical problems which may be responsible for the variability in the data obtained. For example, the yield of the chromosome type of asymmetrical exchanges (ring and dicentric chromosomes) per cell can be roughly fitted to the expression given by $Y = kD^2$, where k is the coefficient of aberration production and where D is dose in rads. Although the data of Bender and Gooch[2] and of Norman *et al.*[3] fitted well to this expectation, those of Evans[4,5] appear to fit best to the expression $Y = kD^{1.16}$. The first aim of the present experiments was to reinvestigate the dose-response relationship in these cells under various conditions of culture. It seemed possible that changes in the culture conditions of human leukocytes, which represent a heterogeneous population, could affect the division time of different irradiated cells with varying radiosensitivity. Sampling of different proportions of the mixed population may conceivably influence the observed aberration yield.

The second aim of the present studies, in a series of dose-fractionation experiments, was to verify the reported "Lane effect" in these cells.[4] When the leukocyte population was irradiated with split doses given at increasing time intervals between fractions, the changing aberration yield could also be due to mitotic delay and scoring cells of variable radiosensitivity.

Freshly drawn blood from two donors was exposed to 250-kv x rays at single doses ranging from 0 to 300 r. After irradiation, an 0.8-ml aliquot of blood was added to 10 ml of culture medium containing 0.2 ml of phytohemagglutinin (PHA, Wellcome). This method is very similar to that of Evans. For the dose-fractionation experiments, it was necessary to irradiate the blood after it had been set up in culture. Thus, in the first experiment of this series, a single dose of 200 or 100 r was given to the cultures immediately after setting up and at varying times up to 24 hr

later. In the second and third experiments either two equal or unequal portions of a total dose of 200 r were delivered. The first dose was always given soon after setting up the culture, while the time of the second dose was varied. Also in these experiments a single dose of 200 or 100 r was given at the same time as the second dose. The blood cells were incubated at 37°C for 54 hr, during the last 5 hr of which colchicine was included in the culture medium. Usually 100 and sometimes 200 cells at every treatment in each experiment were scored for metaphase chromosome aberrations. For the present purposes the frequencies of induced ring and dicentric chromosomes only are discussed. The spontaneous frequency of these types is close to zero.

In order to find the time of DNA synthesis and mitosis in these cultures, a series of cultures were fixed at various times. ^{3}H-Thymidine (2.0 curies/millimole, 1 μc/ml) was added $\frac{1}{2}$ hr before fixation. Colchicine was not used in this experiment. One thousand cells per time period were scored for the labeling and mitotic indices.

Results of two repeat experiments on whole blood irradiated with a single dose prior to culture show that the dose-response curves for dicentrics and rings fit best to the equations $Y = 1.9 \times 10^{-3} D^{1.9}$ and $Y = 8.6 \times 10^{-3} D^{1.4}$ respectively.[6] Similar results were obtained when the blood was irradiated shortly after having been added to the culture medium containing PHA. If, however, the blood was incubated in the presence of PHA for 6 to 24 hr before being x irradiated with 200 r, an overall 10% increase (significant at the 95% level) in the frequencies of ring and dicentric chromosomes was observed, as compared with those cells x irradiated soon after the blood was drawn. With 100 r a borderline increase in the aberration frequencies was also evident. From our labeling studies we know that between 0 and 24 hr only 0.1% of the cells are synthesizing DNA at any one time, and, therefore, over this time period the cells being irradiated were in either the G_0 or G_1 stage. Our efforts to synchronize the leukocyte population either by prolonged incubation without PHA or by treatment with excess thymidine were not successful.

The results of two dose-fractionation experiments are summarized in Fig. 1.17.1. The frequencies of rings plus dicentrics in the cells irradiated with a single dose of either 100 or 200 r during the first 8 hr after the blood was set up in culture are shown. There is a tendency of increasing frequencies at the latter periods, confirming the result discussed above. When cells were given two equal doses of 100 r each separated by varying intervals, the aberration yields fluctuate, but the fluctuation was not as extensive as that reported by Evans.[4] Finally, when cells were given two unequal doses either 150 r + 50 r or 50 r + 150 r, separated by varying intervals, a different pattern of fluctuation in total aberration yields was observed.

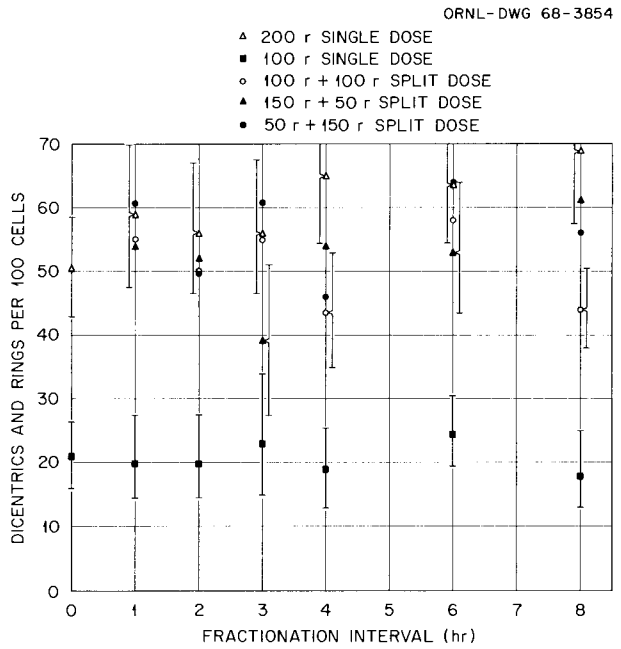

Fig. 1.17.1. The Combined Frequency of Dicentrics and Rings After Split and Unsplit X-Ray Doses.

Under the experimental conditions, the dose-response curves we obtained for rings and dicentrics are similar to those obtained by Bender and Gooch[2] and Norman *et al.*[3] but differ from those by Evans.[4,5] Evans[5] suggested that the possible reasons for these differences in the exponential power of dose curves could be due to the fact that other authors in their experiments had lost a proportion of the abnormal cells during separation of leukocytes before culturing and harvested cells later than 60 hr. We have demonstrated here that these two factors do not affect the shape of the dose-response curve.

It is perhaps interesting to point out that in our data the ratio of dicentric chromosomes to ring

chromosomes is 4.5:1. This ratio is 50% higher than that obtained in *Tradescantia*, possibly because of a larger number of chromosome arms in the human complement.

The observed increase in aberration frequency in cells irradiated with unsplit 200 r after 6 hr in culture could be due to a change in radiosensitivity of the cells as they progress through G_1 toward S. With split doses given at varying intervals, the observed fluctuations in aberration yield could be explained by the fact that the first dose induces mitotic delay and/or cell synchrony. Consequently the second dose given at varying times after the first dose induces aberrations in cells of different sensitivities. This idea is supported by our observation that we can alter the time of the fluctuation by altering the size of the first dose. Furthermore, in hamster cells in culture which have uniform G_1 sensitivity, there was no evidence for the Lane effect.[7]

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1.18 CHROMOSOME PREPARATIONS FROM MOUSE PERIPHERAL BLOOD CULTURED IN VITRO AND IN VIVO

Karin E. Buckton Paul Nettesheim[1]

With the development of a large number of inbred strains of mice over the last two decades, this mammal has become the most favored experimental animal, particularly for genetic studies. However, the cytogenetics of mice has been much less explored, largely because of the difficulties in obtaining chromosome preparations from mouse peripheral blood, which is a readily available tissue for repeated studies.[2] In most species of mammals the leukocytes of the peripheral blood will respond to the stimulus of the mitogen phytohemagglutinin (PHA), transform into "blast" cells, and divide; but in cultures of mouse peripheral blood only inconsistent results have been obtained in this way.

We have succeeded in growing small amounts of mouse peripheral blood in vitro. Our method is a modification of the standard culture technique for obtaining chromosome preparations from human peripheral blood.[3] The pokeweed mitogen was used instead of PHA. At the same time a method was developed for obtaining chromosome preparations from mouse peripheral blood filled into diffusion chambers inserted into recipient rats or mice. No mitogen was necessary in this latter method. The pattern of cell growth in both systems was determined by studying the rates of DNA synthesis and cell division in the transformed large lymphocytes. The peaks of DNA synthesis and mitosis were reached at 52 hr in vitro and 5 to 6 days in vivo after the initiation of culture. Both methods consistently yielded a sufficient number of mitoses suitable for chromosome analysis.

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1.19 THE KINETICS OF X-RAY-INDUCED CHROMATID TERMINAL DELETIONS AND GAPS IN CHINESE HAMSTER CELLS

Sandra L. Bell E. H. Y. Chu

There has been a controversy over the last few years about how ionizing radiations induce chromosome aberrations. One interpretation[1] is the "breakage-first" hypothesis, which states that radiation breaks chromosomes and that these breaks can either remain open or rejoin in one of several ways. The types of aberrations induced

can be grouped broadly into those that are the result of one break and those that require two or more breaks. One-break, or one-hit, aberrations increase linearly with dose; two-break, or two-hit, aberrations increase approximately as the square of the dose.

The alternative hypothesis proposed by Revell[2] is called the "exchange hypothesis." According to Revell, radiation causes chromosome exchanges rather than chromosome breaks. There must be contact between chromosomes before an exchange, and thus an aberration, can occur. Terminal deletions are the result of incomplete exchanges and should follow two-hit kinetics. In theory this should be easily resolvable by obtaining experimental data and expressing the number of terminal deletions as the function of dose. In practice, however, this presents a major difficulty, the reason being that the number of terminal deletions is very low in plant materials, where most studies of chromosome aberrations are undertaken. The problem is further complicated by the occurrence of chromatid "gaps," which are regions of the chromosome where staining does not occur. Since there is no displacement of the chromatid segment distal to the gap, these should not be scored as deletions. Nevertheless, the kinetics of gap production is in itself of additional interest.

In many mammalian cells the number of chromatid terminal deletions is considerably higher than that found in plants.[3] We have, therefore, undertaken experiments designed to determine the frequencies of terminal deletions and gaps that can be induced readily at certain cell stages.

Chinese hamster cells in culture, mitotically synchronized with 5-aminouracil, were x irradiated either at the beginning of the DNA synthesis period (S) or at the postsynthesis period (G_2). Cells were treated with colchicine for 3 hr to accumulate metaphases, exposed to hypotonic saline, fixed, and stained. Two hundred metaphases were scored for the number of true terminal deletions (fragment displaced) and number of chromatid gaps.

In unirradiated controls, 5-aminouracil caused both types of aberrations.[3] Both terminal deletions and chromatid gaps were induced by x rays in high frequencies during G_2 and increased linearly with dose (Table 1.19.1). Therefore they appeared to be the result of a single event, supporting the breakage-first hypothesis. There were fewer gaps and terminal deletions in the cells that were irradiated while in early S. This would seem to indicate that more restitution occurred in these cells either because of the occurrence of DNA synthesis or because of the longer time interval involved between the induction of breakage and the appearance of the aberration at metaphase. The large increase of chromatid terminal deletions could also be due to chromosome movement in preparation for division.

Table 1.19.1. The Number of Terminal Deletions and Chromatid Gaps in X-Irradiated Cells of Chinese Hamster[a]

Dose (r)	No. of Terminal Deletions		No. of Gaps	
	S Period	G_2 Period	S Period	G_2 Period
0	4	8	14	25
50	15	12	24	57
100	9	38	27	102
150	19	54	35	152
200	22	65	35	205
300	28	97	57	273

[a]The results of two experiments are grouped together. A total of 200 cells were scored at each dose.

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1.20 THE DEVELOPMENT AND APPLICATIONS OF VAPOR-FIXATION METHOD FOR VERTEBRATE CHROMOSOME PREPARATIONS

Tatsuo Omura[1]

A new cytological method has been developed for excellent chromosome preparations from a very limited number of animal cells in vivo and in vitro. For in vivo studies, animals are treated with colchicine (10^{-3} *M*) for 3 to 6 hr prior to

sacrifice. Various organs or tissues from the adult or fetuses are removed, and the cells are separated and suspended in a small volume of hypotonic (0.6%) sodium citrate for 10 min on a slide. The chromosomes become spread, and practically all the cells are fixed directly on the slide after its being exposed for 60 to 90 min to the vapor of a fixative (three parts glacial acetic acid, one part ethanol, and four parts distilled water). After brief dipping in absolute and 20% ethanol and in a mixture of one part glacial acetic acid and three parts ethanol, the slide is air-dried and the cells stained with Giemsa.

This method was first tried in the blood corpuscle of the chick embryo, then with the bone marrow cells of the frog, and finally with various mammalian tissues.

To illustrate its applicability, a study was initiated to investigate the pathological immune response (graft vs host reaction) at the chromosome level. Normal, intact mice injected with rat bone marrow cells (100×10^6 cells/mouse) showed, as a rule, no replacement in the host hemopoietic and lymphoid tissues by the grafted cells. In mice x irradiated with 950 r prior to the grafting of rat bone marrow cells (100×10^6 cells/mouse), there was no mitosis seen in the host hemopoietic and lymphoid tissues in the following day (day 1). Beginning on day 2, however, rat metaphase figures were seen with increasing frequencies in the femur and spleen of the host. Rat metaphases were detected in host's mesenteric lymph node at day 6 and in brachial and inguinal nodes at day 9. Most interesting is the fact that in the thymus of the host the only dividing cells that began to appear on day 8 were those of host.

Reference

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1.21 POSSIBLE MODIFICATION OF GENETIC EXPRESSION OF MAMMALIAN CELLS WITH MONOVALENT SALTS

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Changes in ionic environment have been implicated in the control of developmental changes in a number of systems, including *Chironomus*, hydra, and rat epithelium. It has been suggested that such salt effects arise from a direct action on RNA synthesis, perhaps by selective alteration of the DNA-histone interactions at different loci. In order to test this hypothesis directly, mouse L cells in monolayer culture were exposed for various periods to media containing different sodium chloride concentrations. Cold RNA and ^{3}H-uridine-labeled RNA were isolated and hybridized in vitro with DNA prepared from mouse embryos.

Cells were grown at 70, 140 (normal), and 220 m*M* salt, with only slightly depressed growth rates at the extremes. Competition experiments with all possible pair combinations between RNA from cells grown at the three different salt levels disclosed no qualitative differences among them. No differences were noted whether ^{3}H-RNA was prepared from cells grown for three days at each level or from cells transferred on the third day just prior to isotopic labeling from the normal medium to either 70 or 220 m*M*; thus there were neither short- nor long-term effects as measured by this method. In saturation experiments with various classes of RNA, slight quantitative differences have been noted. Metal analyses of cells and isolated nuclei revealed no significant difference in the cellular content of Na, K, Ca, and Mg grown at the three salt levels. Thus within the range of salt level tested and employing the present experimental procedures, no qualitative modification of RNA synthesis in these cells could be achieved.

Reference

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1.22 ISOANTIBODY SEPARATION

Diana M. Popp R. A. Brown[1]

H-2 isoantibodies have been separated by gel filtration and are characterized by 7S and 19S immunoglobulins.[2] The products of gel filtration of serum contain nonspecific antibody and other molecules of similar molecular weights. It is possible to separate specific anti-sheep red blood

cell immunoglobulins by absorption and subsequent elution from sheep red cell stroma.[3] Similar methods were used in an attempt to purify H-2 isoantibodies produced in mice after the injection of allogeneic tissues.

Hyperimmune sera were induced by serial injection of splenic homogenates, and the sera were collected 12 days following the last injection. The stroma was obtained by lysing washed red cells in distilled water, centrifugation at 7000 × *g* for 15 min, and repeated washing of the pelleted stroma in 0.1% NaCl until the supernatant is clear. The stroma is resuspended in phosphate-buffered saline (PBS). Aliquots of the suspended stroma are pelleted and incubated in antiserum for 2½ hr at room temperature and for 48 hr at 5°C. The mixture is brought to 10 ml with PBS made 1% with bovine serum albumin. The stroma is pelleted and washed once with 1% BSA-PBS and once with PBS; 0.2 ml of glycine-sulfate buffer, pH 2.5, is added to the pellet, which is slurried and pelleted. The supernatant is buffered with a ⅕ volume of 1 *M* Tris, pH 10. Red cell agglutination was carried out according to the technique of Gorer and Mikulska.[4]

Titration of eluates from stroma of various strains of mice, Table 1.22.1, shows that stroma from the tissue donor strain yields antibody activity to its erythrocytes and to erythrocytes from other strains of mice which carry common H-2 antigens. Stroma from the strain in which the antibody was made yields no antibody. Stroma from strains which are neither the donor nor recepient, but which carry one or more antigens in common with the donor strain, yields antibody specific for those antigens. The methods permit the removal of a single antibody from a multispecific antiserum. The procedure is quite simple, has a high yield of antibody, and promises to be a useful technique for the genetic and biochemical studies on antigens and antibodies of the H-2 system in the mouse.

A sample of antibody absorbed on and eluted from stroma of donor erythrocytes was subjected to sucrose density centrifugation, and the various cuts were analyzed to determine the physical nature of the isoimmunoglobulins. The greatest hemagglutinin activity appeared in the region of the 7S fraction, and lesser activity appeared in the region of the 19S component. These results are consistent with those of Andersson *et al.*[2]

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Table 1.22.1. Reaction of RBC from Various Strains of Mice to Eluates from Stroma

+ = agglutination
0 = no agglutination

RBC	C3H Anti A/Sn Antiserum Absorbed on Stroma of Strains				RFM Anti A/Sn Antiserum Absorbed on Stroma of Strains		
	A/Sn, H-2[a]	BALB/c, H-2[d]	RFM, H-2[f]	C3H, H-2[k]	A/Sn, H-2[a]	RFM, H-2[f]	C3H, H-2[k]
A/Sn, H-2[a]	+	+	+	0	+	0	+
BALB/c, H-2[d]	+	+	+	0	+	0	+
DBA/2, H-2[d]	+	+	+	0	+	0	+
A.SW, H-2[s]	+	+	0	0	+	0	+
C3H, H-2[k]	0	0	0	0	+	0	+
RFM, H-2[f]	0	0	0	0	0	0	0
C57BL, H-2[b]	0	0	0	0	+	0	0

1.23 DESIGNATION OF HISTOCOMPATIBILITY-14 LOCUS IN THE MOUSE

Diana M. Popp George D. Snell[1]

Preliminary studies indicate that the red cell agglutinogen designated "rho"[2] and the R and Z antigens[3] are products of the same or closely linked loci. A more detailed study was undertaken to establish the correlation, to assign a common locus designation to the factors, and to investigate the effect of the locus on tissue transplantation.

Inbred strains of mice obtained from Bar Harbor and maintained in this laboratory were used. An (A.CA × RFM)F_2 population was produced to study the genetic segregation of the factors. A congenic line, C57.F, established in this laboratory was used for the skin grafting experiments. The line was at generations $BC_{15}F_2$, F_3, or F_4 at the time skin grafts were exchanged. The inbred strain C57/Ha and the congenic line C57.F differ at the rho or R-Z locus, now called *H-14*; the source of the $H\text{-}14^a$ allele is strain F/St. Four antisera were supplied by Dr. Snell: RIII/J anti C3H, RF/J anti C3H, (RF/J × RIII/J) anti C3H (anti-Z), and LP.RIII anti RIII/J (anti-R). Antiserum A.CA anti RFM (anti-rho) was produced in this laboratory. Agglutinations were carried out according to the method of Gorer and Mikulska.[4] In vitro absorptions were carried out on saline-washed tissue homogenates, and in vivo absorptions were accomplished by the injection of 0.1 ml of antiserum intraperitoneally, and the serum was recovered 18 hr later. Tail skin grafts were exchanged, and immunization of graft recipients was accomplished by the injection of thymus or packed erythrocytes ten days prior to grafting.

The identity of rho and R factors and the allelism of rho and Z were established by the analysis of 189 (A.CA × RFM)F_2 mice. Although the population did not reveal an expected 1:2:1 ratio for the segregation of two alleles, all animals which reacted with anti-rho serum also reacted with anti-R serum. Moreover, all animals which did not react with anti-rho also did not react with anti-R. There were no animals which were negative for all three sera, indicating that at least one or the other allele or both occurred in all progeny.

A unique tissue distribution for the rho factor has been established[2] and shows that the antigen can be found in spleen, liver, erythrocytes, brain, lung, testes, kidney, and thymus. Tissue absorption experiments indicated that R and Z have the same tissue distribution as rho. In addition, strain RFM tissues remove anti-R activity for strain RIII, RIII tissues remove anti-rho activity for RFM, and A.CA removes anti-A activity for C3H. In vivo absorption also shows that strain A.Ca clears activity for 20 other strains of mice, and in vivo absorption in C57BR clears activity for 10 strains tested. These data suggest that, unlike H-2, this is a single-factor system. Tail grafts were exchanged between C57.F, homozygous for the Z factor. No rejections were seen in unimmunized recipients within 120 days, or in recipients immunized by erythrocytes or thymus within 90 days after grafting; these grafts will be followed further.

Although skin graft rejection was not demonstrated, a circulating antibody induced after tissue injection indicates incompatibility at this locus. Tissue absorption studies also indicate the presence of the antigen on tissues other than erythrocytes. Such evidence is ample to place this locus in the histocompatibility series. The symbol *H-14* identifies the locus; allele $H\text{-}14^a$ designates the factor previously known as rho or R, and allele $H\text{-}14^b$ designates the factor previously known as Z. Identification of transplantation antigens not expressed in skin but expressed on other tissues is not an unlikely or new phenomenon but does demonstrate the importance of tissue-specific antigens. When considering a tissue transplant, not all antigenic differences must be reckoned with but only those which are represented on the tissues under consideration.

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1.24 PRIMARY STRUCTURES OF THE α CHAINS OF HEMOGLOBINS FROM C3H AND C3H.B MICE

K. M. Hilse J. E. Attrill[1]
R. A. Popp W. S. McEwen

Solubility in phosphate buffers has been used as a criterion to detect hemoglobin differences

among inbred strains of mice.[2] Amino acid substitutions in the α chains of the hemoglobin molecule are associated with the solubility differences observed for mouse hemoglobins; this fact has been established by determining the primary structures of the α chains of hemoglobins from C57BL, NB, and BALB/c mice.[3] A fourth hemoglobin, namely that in C3H.B mice, has a solubility distinct from that of the three cited above, and solubility studies also suggested that C3H hemoglobin was very similar to, yet distinct from, that of either NB or C57BL.[2] C3H.B was established as a subline of C3H/St when a recessive black-eyed, white-coat point mutation arose in the C3H/St strain. The primary structures of the α chains of these two strains are being compared with one another and with the three α chains analyzed earlier.[3]

The preparations of hemoglobin, globin, α chain, and tryptic peptides were as previously described.[4] The tryptic peptides were separated by chromatography on Dowex 50-X2 (1.9 × 56 cm) at 55°C using a stream divider assembly on a model 120C amino acid analyzer. Purity of peptides was checked by paper chromatography, and fractions from the Dowex 50-X2 column that contained more than one peptide were further purified on columns of Dowex 1-X2. The amino acid content of each tryptic peptide was determined, and the sequence of amino acids was determined by Edman degradation.[5]

The amino acid analyses of the tryptic peptides suggested that C3H.B α chain has the same primary structure as the α chain of C57BL hemoglobin with one exception. Whereas αT-9 is homogeneous in C57BL mice, the data indicate that this region of the α chain of C3H.B mice exists in two forms. Edman degradation showed that equal quantities of asparagine and serine are present at position 68 of the α chain of C3H.B hemoglobin. "Duplexity" is used to describe the state in which two amino acids are found at the same position in alternate forms of the same protein in a homozygous individual or individuals of an inbred strain. Similar studies on C3H α chain showed that αT-4 exists in two forms and that αT-9 exists in two or perhaps four forms. In the α chain of C3H hemoglobin, peptide αT-4 has either the amino acid glycine or valine in position 25. This amino acid substitution alters the chromatographic behavior of the tryptic peptide so that each component appeared in a separate fraction from the Dowex 50-X2 column. Each fraction was subsequently processed differently, so that the absolute quantities of the two forms of αT-4 are uncertain at present. However, the valine-containing peptide was recovered in larger quantities. αT-9 is a large peptide containing 29 amino acids. The amino acid composition of αT-9 showed approximately a half unit of valine and a half unit of isoleucine, four and one-half units of aspartic acid, and two and one-half units of serine. The half units of valine and isoleucine result from relatively equal quantities of these amino acids at position 62, and the half units of aspartic acid and serine result from similar amounts of the amino acids asparagine and serine at position 68. If the heterogeneity of the α chain of C3H hemoglobin results from ambiguous codons, αT-9 should appear in only two forms. The appropriate fragments of αT-9 are presently being isolated to establish whether two or four forms of this peptide exist. Finding four forms of αT-9 would support the hypothesis that the duplexities at positions 62 and 68 result from translational variations during protein synthesis. However, the postulation that these duplexities result from the presence of two α chain cistrons would be supported by finding only two forms of αT-9 in which valine always occurs with asparagine and isoleucine with serine and if reanalysis of αT-4 shows equal quantities of glycine and valine at position 25. Presumably, cistron duplication would then also be the basis for duplexity of amino acids in the α chains of other strains of mice as well.

It should be emphasized that the α chains of C3H mice may represent a combination of two distinct, homogeneous α chains which are also found in strain C57BL and NB mice. Moreover, C3H.B α chains represent a combination of the α chains found in C57BL mice and one of the two forms of α chains found in BALB/c mice. Among the five types of α chains (one defined as standard plus four variants) which have been examined to date, position 25 has twice been found to show amino acid alterations (once a replacement and once a duplexity but both involving glycine and valine); twice position 62 has shown amino acid alterations (once a replacement and once a duplexity but both involving valine and isoleucine); and amino acid alterations have been noted at position 68 in each of the four variants studied (once a replacement of asparagine to serine, once

a duplexity of serine and threonine, and twice a duplexity of asparagine and serine). The interdependent genetic and biochemical bases for these observations are being studied as a basis for understanding protein polymorphism in mammals, of which the N-terminal region of immunoglobulins represents an extreme example.

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1.25 TRANSPLANTATION ANTIGENS OF MOUSE SPLEEN CELLS

R. A. Popp — Diana M. Popp
N. G. Anderson[1] — W. S. McEwen

Subcellular particles that contain the principal transplantation (H-2) antigens of mouse splenic tissue have been separated by zonal centrifugation.[2] Some of the particulate-bound antigen can be solubilized by autolysis or papain.[3] The physical properties of the soluble antigens are being studied to determine whether the multiple specificities of the H-2 alleles are expressed as (1) separate molecules, each molecule having a single specificity; (2) a polymer of separate molecules, each monomer having a single specificity; (3) regional prosthetic groups covalently bound to a protein backbone of a nonantigenic molecule; or (4) specific segments of a single molecule, in which case a specific region comprises a single antigenic site.

The soluble phase obtained by autolysis of the antigenic particles in 0.05 *M* Tris-HCl buffer was passed through Sephadex G-200. Two well-resolved fractions were obtained; the first contained molecules of molecular weight greater than 100,000 and the second contained molecules of molecular weight less than 50,000. Hemagglutination inhibition assays for several of the multiple specificities of H-2 allele showed that antigen specificities H-2.1, 3, 5, and 11 were located in the soluble phase of C3H splenic extract. Three electrophoretically distinct proteins were found in the soluble antigen preparation after gel filtration on Sephadex, but the active component(s) has not been positively identified. The antigenic particles are rapidly solubilized by papain digestion, but the recovery of H-2 activity from the papain-solubilized preparations has not as yet been analyzed.

Purification of the electrophoretically distinct fractions will be required before the biochemical and biophysical characteristics of the antigen can be defined. However, the presence of several antigenic specificities in molecules of molecular weight greater than 100,000 suggests the possibility that the multiple specificities of the H-2 antigens may be located in the same molecule. The recombination frequency of 0.36% for the more distal (H-2.4 and 11) regions of the H-2 locus[4] suggests that these specificities could be located in the distal segments of a single protein which has a minimum molecular weight of 66,000. This limitation is satisfied by analyses that the antigens are found in molecules of molecular weight greater than 100,000. The physical architecture of the H-2 molecule(s) or polymer(s) dictates the approach to analyze the molecule further. If all the major histocompatibility antigens are located on the same molecule, the effort required to obtain material for tolerance experiments will be much reduced. Furthermore, one can be reasonably sure that all the antigens are represented in the material used to produce tolerance. Information to date suggests that the multiple specificities of the HL-A histocompatibility antigens of man are homologous to the H-2 antigens of the mouse. Thus studies on the H-2 antigens in mice will serve as a model for experiments which will be necessary to evaluate the possibility of producing low-dose tolerance in man. A combination of histocompatibility matching through serological typing and of inducing low-dose tolerance through injection of purified antigen may greatly reduce the paramount dangers of graft rejections owing to histoincompatibility which thwarts the extreme skills of surgeons today.

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1.26 MAJOR DEVELOPMENTAL EVENTS IN THE *RHYNCHOSCIARA* LIFE CYCLE

Ellen Mattingly C. L. Parker

The virtues of the sciarid fly *Rhynchosciara* for use in studies in cytology and developmental biology are well known. The one serious disadvantage to the routine use of these flies was that they could not be maintained for extended periods in the laboratory. This report will describe briefly a new culture method that allows the maintenance of unlimited numbers of cultures with relative ease and will present the detailed information now available on the characteristics of the entire life cycle.

Individual cultures, with as many as 500 larvae, are kept in disposable petri dishes on cornmeal agar. The agar is prepared in the laboratory by suspending yellow cornmeal in nonnutrient agar. Commercially available cornmeal agar cannot be substituted. The larvae are placed on the agar with a mass of highly fermented plant material. Either orchard grass or corn ensilage, refermented in the laboratory, makes an excellent food supply. At the end of the larval stages, when the members of a group begin to form a cocoon, they are placed on damp peat moss, where they go through the entire pupal period.

Several advantages, other than those of general ease of maintenance, result from these new culture techniques. Among these is the fact that the larvae can be kept for as long as two weeks on cornmeal agar alone, and during this time they ingest the agar and appear to be able to digest the cornmeal. It is now feasible to consider studies of the effects of various chemical mutagens on the fly by simply maintaining the larvae on agar in which the various chemicals have been dissolved.

An additional practical advantage is that the larvae, when allowed to form their cocoon on peat moss in a plastic petri dish, generally form the cocoon on the lid of the dish. This results in a cocoon in which the larvae nearest the plastic do not form a complete cell. The investigator, therefore, can determine precisely what physiological stage, and even which puff on which salivary gland chromosome, is present, merely by inspection of the petri dish. One can therefore easily and with absolute accuracy accumulate as much as 15 or 20 g of larvae which are all in precisely the same stage of development.

Since the development of the above culture method, the details of the larval life cycle have been described. The female fly lays a mass of eggs, all of the same sex, and then dies. The eggs then develop for approximately 12 days, at the end of which all hatch synchronously. The first three larval molts occur at days 6, 12, and 18. The fourth larval instar, which begins on day 18, is the most variable in length; generally, on about day 40 the larval cocoon begins to form. Approximately five days later, the prepupal molt occurs, and within one or two days the salivary glands lyse. Pupation itself lasts about five days, after which the adults eclose and mate. Within two to three days the female lays her eggs, and both males and females die within five days after eclosion.

1.27 SPERMATOGENESIS IN LARVAL *RHYNCHOSCIARA SP.*

C. L. Parker Ellen Mattingly

The chief characteristics of the life cycle of *Rhynchosciara sp.* have been described above. It is now possible to relate specific stages in spermatogenesis to specific stages of larval development. The nature of this relation will be described first, and a description of variations in incorporation of thymidine-^{3}H at different stages will follow.

By day 20 after the larvae have hatched from the eggs, the testis may easily be dissected from the surrounding fat body. Testes fixed in 3:1 fixative and smeared in acetocarmine stain show no sign of organization of the spermatogonia into cysts. Interspersed among the spermatogonia are large cells with polytene chromosomes. From day 20 to day 30, large numbers of mitotic cells may be found. The cells of any particular stage, for example, metaphase, are not randomly distributed throughout the smear. Rather, groups of from 10 to 20 cells will be in the same mitotic stage.

After day 30, no more mitotic divisions are seen. The chromosomes of the spermatogonia again become dispersed, with the exception of the large limited chromosome, which remains heterochromatic and closely associated with the nuclear membrane. At this time small granules appear in the cytoplasm. During the next ten days, these increase in both size and number. These granules persist through the subsequent meiotic divisions and appear to fuse during the elongation of the sperm.

Meiosis I and II occur during two days early in the cocoon formation stage. It appears that meiosis in the male generally occurs at the same time that the DNA puffs arise in chromosome B in the male. Elongation of the sperm is almost complete at the time the prepupal molt occurs.

The various stages of development in the testes correlate well with differences in incorporation of thymidine-^{3}H. Thus, from day 20 to day 30, intense incorporation is seen in localized areas that correspond to areas in which the nuclei are in interphase. If the larvae are held for 8 hr after injection of the isotope, label may be seen over metaphase chromosomes. In the latter case, in some areas only the limited chromosome is labeled, while in other areas all the metaphase chromosomes except the limited show incorporation.

After day 30, while the cytoplasmic granules are forming, no incorporation of thymidine-^{3}H occurs in the spermatogonia, although the large cells with polytene chromosomes continue to synthesize DNA. This general picture remains constant up to the time that the adults eclose.

1.28 VARYING EFFECTS OF X RADIATION DURING THE LIFE CYCLE OF *RHYNCHOSCIARA ANGELAE*

Ellen Mattingly

Mutants of the sciarid fly *Rhynchosciara* would be useful in studies of the unusual genetic system of this fly. Preliminary attempts to induce mutants by x radiation show that *Rhynchosciara* is in general very resistant to x radiation and that sensitivity varies widely with the stage of the life cycle during which the radiation is given.

Since all the progeny of a single female develop synchronously and are of the same sex, it was possible to select groups of either sex at any stage of development; each group has at least 100 larvae. For these preliminary experiments six stages during the life cycle were chosen: (1) 25 days after hatching (very early in the fourth instar), (2) day 1 of cocoon formation, (3) meiosis I and II in spermatogenesis and corresponding ages in females, (4) one day prior to the prepupal molt, (5) after the prepupal molt, and (6) adults. Doses of x radiation ranging from 500 through 100,000 r were given, and the cultures were subsequently checked for eclosion and subsequent fertility of the adults. Because the life cycle in this fly is approximately 70 days, complete data on fertility and possible appearance of effects in the F_2 and subsequent generations are not complete.

All groups of larvae exposed to either 500 or 1000 r showed normal eclosion rates. Male larvae irradiated in late fourth instar when the cocoon was forming (a stage at which the spermatogonia are in meiotic prophase) showed many chromosomal fragments in the spermatogonia when they subsequently entered meiotic metaphase.

Some higher doses have also been given at various stages. When larvae of either sex are exposed to doses of 10,000 r or more on the day prior to the prepupal molt, no adult flies are obtained. Male pupae and adults, however, have been exposed to doses of 25,000, 50,000, and 100,000 r. The adults survived, and the pupae showed normal eclosion rates. All were subsequently mated with unirradiated females, and the resulting eggs were checked for hatchability. Both of the groups receiving 100,000 r and the adult males receiving 50,000 r were sterile. The male pupae, however, that received 50,000 r were fertile, the resulting eggs showed normal hatchability, and the progeny larvae went through all the larval molts exactly on schedule and appear to be progressing normally through pupation. Salivary glands from about 50 of these F_1 larvae have been checked for the presence of chromosome aberration; none have been seen.

1.29 VARIATIONS IN NUCLEOLAR ACTIVITY AND RNA SYNTHESIS DURING DEVELOPMENT IN *RHYNCHOSCIARA*

Ellen Mattingly Ronald Eckhardt

The nucleolus of *Rhynchosciara* appears in various morphological forms. Reference is made

in the literature to a dispersed state (Pavan, 1966). In the present experiments, we have also found other forms. In addition to the dispersed state, we have found stages of differing degrees of nucleolar accumulation.

We have followed sequential changes in the chromosomes and the "X-associated" nucleolus. Morphological characteristics of the nucleolus were obtained from paraffin sections and squash preparations. RNA synthesis was followed with tritiated uridine and autoradiography.

Although much time has been spent studying the early period of days 1 to 18, the small size of the cells and especially the small size of the nuclei make a description of the cycle of changes difficult. It has been noted, however, that there are morphological changes such as periodic condensation and enlargement of the nucleolus and blebbing at the molts, which are indicative of changes in nucleolar activity. Also it seems that the development of the nucleolus varies from tissue to tissue; in the youngest larvae, the nucleolus appears to function sooner in intestinal cells than in other tissues.

Autoradiographic analysis of RNA synthesis shows a parallel situation. During the early period of larval life, synthesis decreases before each molt and then increases immediately after the molt. The synthesis then gradually decreases until after the next molt.

During the fourth instar, the cycle is basically the same as the previous instars. Here, however, the cycle is drawn out and without abrupt changes in synthetic rate. Immediately after the third molt, activity increases for several days and then gradually falls off. During most of this period the nucleolus appears rather disperse.

During the terminal period of larval life (approximately ten days before the prepupal molt), the clearest picture of the nucleolus may be seen. Initially during this period, a gradual accumulation of nucleolar material is seen, and it seems to reach its peak, at least in the salivary glands, at the time of preliminary net formation. This accumulation can be seen for several more days and then gradually falls off to the time the prepupal molt occurs and subsequent lysis of the gland. During this period, the other tissues consistently seem to be rather inactive in terms of RNA synthesis and nucleolar activity in comparison with the salivary gland.

The morphological picture of the nucleolus and the autoradiographic analyses seem to correspond. We are now beginning to study the various morphological states with the electron microscope. We hope to find a correlation between nucleolar activity and changes of ribosomal activity in the cytoplasm.

1.30 HETEROZYGOUS PUFFS AND BANDS IN *SCIARA OCELLARIS* COMSTOCK (1882)

A. L. P. Perondini Crodowaldo Pavan

In the larvae of *Sciara ocellaris*, the polytene chromosomes of the salivary gland have very large puffs, many of which produce metabolic DNA during development. Morphological differences between homologous bands or groups of bands can sometimes be observed in cytological preparations; in *S. ocellaris* there are several heterozygous bands. In strains of this species collected in the greenhouse of the Biology Division, we have found in the chromosomes of the salivary gland some of the heterozygous bands described by Metz, several others that he did not describe, and two minor inversions, as well as cases in which homologous bands undergo their puffing cycles asynchronously. In the last case, one side puffs and condenses a short time before the other in such a way that at the beginning and/or end of the puffing process the puffed region appears asymmetrical. We think that puff asynchrony is good morphological evidence of different action of some allelic genes in heterozygotes; the heterozygous bands are good examples of how chromosomes may differentiate and may also be an indication of how compound loci may be developed, and this may be a type of chromosomal divergence which plays an important role in dipteran evolution.

1.31 MODEL NUCLEOPROTEINS

D. E. Olins

DNA of eucaryocyte nuclei is characteristically associated with a complex mixture of basic proteins (i.e., histones and, less frequently, protamines). It is generally assumed that these basic proteins function to: (1) modulate DNA packing and conformation and (2) assist in mechanisms for control of RNA synthesis and gene expression.

The purpose of the investigations within this laboratory is to determine "where" and "how" these basic proteins interact with DNA, as a necessary prerequisite to an exact definition of their function.

For this purpose we have concentrated on the physical chemical properties of mixtures of DNA with synthetic basic polypeptides, as models for the native-DNA–basic-protein complexes. The presence of basic polypeptides on native DNA results in a marked stabilization of the helix against thermal denaturation. Furthermore, there is evidence suggesting that certain types of basic side chains preferentially interact with regions on DNA (e.g., poly-L-ornithine and poly-L-lysine exhibit preferential stabilization of AT-rich regions). The length of the basic polypeptide is of great importance in determining the character of the DNA-polypeptide interaction. Increasing the number of residues in a series of oligolysines converts the character of stabilization, as measured by thermal denaturation experiments, from a "dynamic" type (i.e., similar to the effects of Na^+, Mg^{2+}, spermines, and spermidines on DNA) to a more "irreversible" type (i.e., a biphasic DNA melting profile at cation/DNA phosphate ratios less than 1.0). Of particular interest in comparison with the studies of DNA–basic polypeptide are the observations that certain histone fractions and, particularly, the protamines, when mixed with native DNA, reveal thermal denaturation profiles similar to those observed with oligopeptides. It thus appears that the type of interaction observed between basic polypeptides, histones, or protamines and DNA, as viewed in terms of stabilization against thermal denaturation, is a complex function of: (1) preferential interactions between basic side chains and regions on DNA and (2) the clustering and spacing of basic residues along the peptide chain.

Present studies are focused on determining whether or not other mechanisms exist to control specific interactions between basic proteins and regions on DNA.

2. Fungal Genetics

F. J. de Serres

Radiation Mutagenesis (2.1, 2.2)

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Participation in Cooperative Programs:

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AEC-NASA Space Biology (26.58)

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2.1 DOSE-RATE EFFECTS ON THE SPECTRUM OF RECESSIVE LETHAL MUTATIONS AT SPECIFIC LOCI IN *NEUROSPORA CRASSA*

F. J. de Serres H. V. Malling
B. B. Webber

Previous studies[1,2] have shown that the recessive lethal mutations induced by x rays at two specific loci in the *ad-3* region (*ad-3A* and *ad-3B*) result from both point mutations and chromosome deletions. The former class of mutation increases in proportion to x-ray dose, whereas the latter class increases as the square of the x-ray dose. These same studies[2] also showed that the single-hit events that resulted in these two classes of mutation were quantitatively different and that since point mutations are not converted to chromosome deletions at very high radiation exposures, they are most probably qualitatively different.

To explore this latter point more thoroughly a dose-rate experiment has been performed to determine whether a reduction in dose rate from 1000 r/min to 10 r/min at room temperature would provide an opportunity for repair that would affect one type of lesion but not the other.[3] It is well known that the frequency of two-hit chromosome

aberrations can be altered by changing the rate of radiation exposure, but no comparable study exists in a system where specific locus recessive lethal mutations can be characterized so completely.

The effect of variation in dose rate on survival and overall forward-mutation frequencies at the *ad-3* loci was discussed in a previous report.[3] The genetic tests to determine genotype and to distinguish point mutations from chromosome deletions have only recently been completed.

The dose-response curves for point mutations (Fig. 2.1.1) show that there is no effect of a decrease in dose rate from 1000 r/min to 10 r/min on the frequency of this class of event. All three sets of data give a good fit to a curve with a slope of 1.0. It is also of interest that the saturation in the dose-response curves with the 40-kr exposure was found in both 1000-r/min treatments but not with the 10-r/min treatment.

The dose-response curves for chromosome deletions (Fig. 2.1.2) show that there is no significant difference between the two 1000-r/min treatments. The initial part of each curve gives a good fit to a curve with a slope of 2.0, as expected, but since saturation was found with the 20- and 40-kr exposures, we get an even more pronounced departure from expectation with this class of recessive lethal mutation than with point mutations. Much lower forward-mutation frequencies were found with the 10-r/min exposures than with the 1000-r/min exposures. The dose-effect curve for the 10-r/min exposures is complex and appears to be a composite of a one-hit and two-hit curve. This experiment shows clearly that there is a

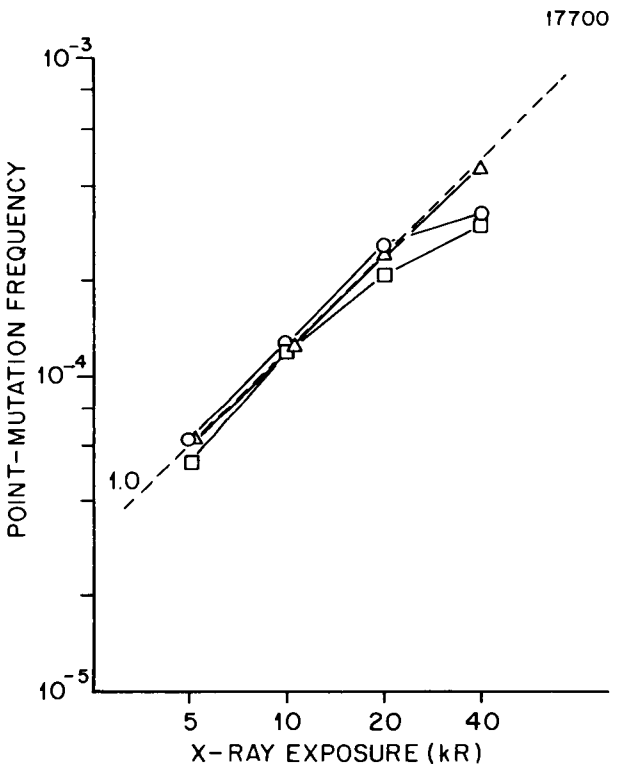

Fig. 2.1.1. Frequency of Recessive Lethal Mutations Resulting from Point Mutation ($ad\text{-}3^R$) in the *ad-3* Region in Heterokaryotic Conidia of a Dikaryon of *Neurospora crassa* at 1000 and 10 r/min. ○ = 1000 r/min plus 66 hr postirradiation storage, □ = 1000 r/min plus no postirradiation storage, Δ = 10 r/min; dashed line = theoretical curve with a slope of 1.0.

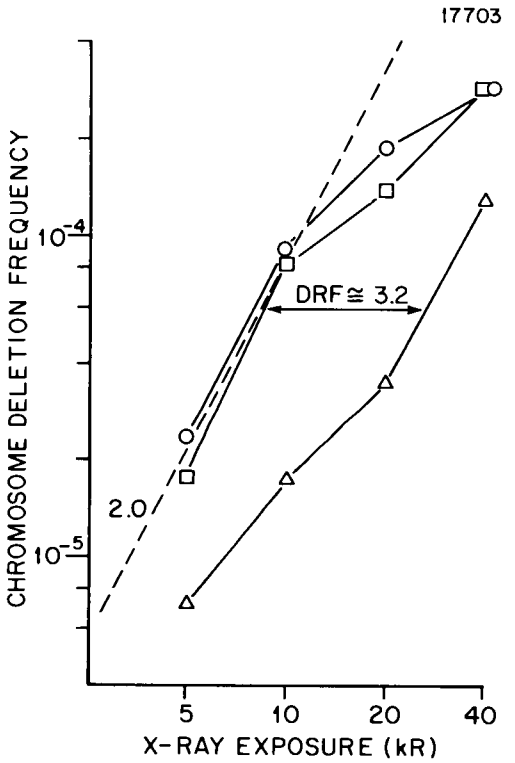

Fig. 2.1.2. Frequency of Recessive Lethal Mutations Resulting from Multilocus Deletion ($ad\text{-}3^{IR}$) in the *ad-3* Region of *Neurospora Crassa* at 1000 and 10 r/min. ○ = 1000 r/min plus 66 hr postirradiation storage, □ = 1000 r/min plus no postirrodiation storage, Δ = 10 r/min; dotted line = theoretical curve with a slope of 2.0.

pronounced dose-rate effect on those recessive lethal mutations resulting from multilocus deletion with a reduction in the rate of exposure from 1000 r/min to 10 r/min. The forward-mutation rate for the single-hit events that cooperate in pairs to produce mutation by multilocus deletion drops from 9.22 to 2.94×10^{-7} mutant survivor^{-1} r^{-1}.

From the present experiment we have concluded that the x-ray-induced lesions that produce point mutation at specific loci are qualitatively different from the lesions that produce chromosome breakage, because the former class does not respond significantly to a change in dose rate, whereas the latter class shows a marked decrease in frequency when the dose rate is lowered.

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[3]F. J. de Serres, *et al.*, *Biol. Div. Semiann. Progr. Rept. July 31, 1965*, ORNL-3853, pp. 54–57.

2.2 THE INFLUENCE OF ANOXIA ON X-RAY-INDUCED RECESSIVE LETHAL MUTATION IN *NEUROSPORA CRASSA*

F. J. de Serres

The effect of anoxia on the frequency and type of x-ray-induced recessive lethal mutation at specific loci was studied after a 2500-rad exposure (250 kvp, 30 ma, 3 mm Al added filtration, 25°C) on a conidial suspension of a genetically marked two-component heterokaryon of *Neurospora crassa*. The comparison was made between conidial suspensions bubbled with air or nitrogen prior to irradiation. Oxygen levels were monitored throughout the irradiation as well as the pre- and post-irradiation phases of the experiment with a Beckman model 160 physiological gas analyzer fitted with a microelectrode. The treated suspensions were assayed for survival of the heterokaryotic fraction and for the induction of recessive lethal mutations at the *ad-3A* and *ad-3B* loci.

The percentage survivals of the heterokaryotic conidia and the overall forward-mutation frequencies among survivors were 100% and 16.0×10^{-6}, respectively, in nitrogen and 94.3% and 38.3×10^{-6}, respectively, in air. These data show that anoxia gives protection against both inactivation and induction of forward mutations by x rays.

The specific locus mutants were characterized by means of genetic tests to determine genotype, to distinguish point mutations from multilocus deletions, and to obtain a tentative identification of the genetic alterations in the point mutations at the *ad-3B* locus at the molecular level. These tests have shown that the reduction due to anoxia in frequency of one-hit events leading to point mutations was not different from the reduction in one-hit events leading to multilocus deletions. This result is in contrast with the results from a previous aerobic experiment[1,2] with two different x-ray exposure rates (1000 r/min and 10 r/min), which showed that single-hit events leading to multilocus deletions were repaired at 10 r/min, while point mutations were not, implicating two qualitatively different events. With anoxia, the reductions in frequencies of the different types of multilocus deletions were correlated with their size, that is, $(ad\text{-}3A\ ad\text{-}3B)^{IR} > ad\text{-}3B^{IR} > ad\text{-}3A^{IR}$. Furthermore, with anoxia no difference was found in the frequency of *ad-3B* point mutations with polarized complementation patterns, but a 60% reduction was found in the frequency of noncomplementing mutants and of mutants with nonpolarized patterns.

These data show that the two major classes of recessive lethal mutations, that is, point mutations and multilocus deletions, are reduced in overall frequency to the same extent by irradiating under anoxic conditions but that with anoxia the mutant spectra within each of these two classes are different from those in air. It is possible that the effect of anoxia on the spectrum of chromosome deletions is an effect on recovery, whereas the effect on point mutations is a more direct effect. It seems likely that chromosome movement during a longer period of recovery under anoxia would lead to a more extensive loss of those multilocus (chromosome) deletions due to pairs of chromosome breaks located some distance apart than of those located in close proximity to each other. The effect on the spectrum of complementation patterns in point mutants most probably results from a shift in the type of damage to DNA as a consequence of of irradiation under anoxia, which, in turn, results in a different spectrum of genetic alterations at the *ad-3A* and *ad-3B* loci.

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2.3 IDENTIFICATION OF THE GENETIC ALTERATION IN SPONTANEOUS MUTANTS AT THE *ad-3A* AND *ad-3B* LOCI OF *NEUROSPORA CRASSA*

H. V. Malling F. J. de Serres

Spontaneous mutations are the raw material of evolution. To obtain some understanding of the mechanism of gene evolution it is important to identify the genetic alterations that can take place spontaneously at the molecular level. We have attempted to identify the genetic alteration in spontaneous *ad-3* mutants by testing their revertibility after treatment with specific mutagens such as nitrous acid (NA), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), *O*-methylhydroxylamine (OMHA), and a monofunctional acridine mustard derivative (ICR-170). The mutagenic specificity of the compounds has been discussed in previous papers.[1,2]

The spontaneous forward mutation frequency in the *ad-3* loci is low ($\sim 0.3 \times 10^{-6}$ per survivor); therefore only a total of 31 mutants have been accumulated during the last years in the *Neurospora* wild-type strain OR74A. Four mutants were excluded from the analysis because they were too leaky on minimal medium or because of their high spontaneous mutability. The genetic alteration in the remaining 27 mutants was determined by following the rules given below.

1. Base-pair transition mutants with GC at the mutant site are characterized by reverting with OMHA. These mutants may or may not revert after treatment with any of the other mutagens.
2. Base-pair transition mutants with AT at the mutant site are characterized by reverting with either NA or MNNG but not after treatment with OMHA.
3. Base-pair insertion or deletion mutants are characterized by reverting after treatment with ICR-170, and they do not revert after treatment with NA, MNNG, and OMHA. Some of these mutants may revert after treatment with EMS.
4. Unidentified genetic alterations: Mutants exist which only revert spontaneously but not after treatment with NA, MNNG, EMS, OMHA, and ICR-170. The possible types of genetic alterations in such mutants have been discussed in detail in previous papers,[1] and we concluded that some of the mutants may arise from transversions.

By applying the above set of rules to the results of the revertibility test we determined the genetic alteration of the spontaneous mutants (the results are summarized in Table 2.3.1). There was no difference between the spectrum of the genetic alterations in the *ad-3A* and the *ad-3B* locus. In total (Table 2.3.1), 70% of the mutants had a base-pair transition at the mutant site, of which 33% of the total had GC at the mutant site and 37% of the total AT at the mutant site; 15% arose from base-pair insertions or deletions; 11% reverted only spontaneously; and 4% did not revert at all.

Very little is known about the mechanism of spontaneous mutation. The distribution of spontaneous mutations in the rII locus of bacteriophage T4 shows that there are certain spots in the gene which mutate extremely more frequently than

Table 2.3.1. Spectrum of Genetic Alterations Within Spontaneous Mutations in the *ad-3* Loci in *Neurospora crassa*

Type of Mutant	No. of Mutants			Frequency (%)
	ad-3A	*ad-3B*	Total	
Identified				
GC	4	5	9	33
AT	3	7	10	37
+/–	0	4	4	15
Nonidentified				
SP	1	2	3	11
NON	0	1	1	4

Abbreviations: GC = guanine-cytosine; AT = adenine-thymine; +/– = base-pair insertion or deletion; SP = mutants which reverted spontaneously but not after treatment with NA, MNNG, EMS, OMHA, and ICR-170; NON = nonreverting mutants.

others ("hot spots"). The occurrence of a spontaneous mutation may depend not only on the base pair involved but also on the electrostatic environment, the neighboring base pairs, etc., and this may explain the appearance of "hot spots." The spectrum of genetic alterations in the spontaneous mutations in the rII locus in phage varies with the conditions under which they are isolated. In bacteriophage T4 about 50% of the mutants were base-pair transitions in the experiments of Drake and McGuire,[3] whereas there were only 14% base-pair transitions in the experiments of Freese.[4]

There is no reason to expect the same type of mechanisms to account for spontaneous mutation if they arise mainly during DNA replication in different organisms. But it is important to extend the study of spontaneous mutation from procaryotes to eukaryotic systems, such as *Neurospora*, where the chromosome organization is similar, if not identical with higher diploid forms, where such analyses are more difficult technically. In this connection, a similar study is in progress with those spontaneous *ad-3* mutations recovered in a two-component heterokaryon, where recessive lethal mutations resulting from multilocus (chromosome) deletions can be studied as well as point mutations.

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3. Drosophila Cytology and Genetics

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Biochemical Genetics (3.1–3.3)

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Mary S. Uziel
Elizabeth S. Von Halle[c]

Chromosome Behavior (3.4–3.12)

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J. R. Merriam[d]
D. L. Lindsley
C. W. Hinton[c]
I. Lucile Norton[e]
Eileen J. Slaughter[c]
J. W. Day

Radiation Genetics (3.13–3.18)

D. R. Parker[f]
J. H. Williamson[g]

[a]Effective August 31, 1967, Dr. Lindsley left the Biology Division to accept a position with the Department of Biology, University of California at San Diego, La Jolla, California. Dr. E. H. Grell was appointed to the position vacated.

[b]In August of 1967, Dr. E. H. Grell was appointed to the position vacated by Dr. Lindsley.

[c]Consultant
[d]USPHS Postdoctoral Fellow
[e]Dual assignments
[f]NSF Senior Postdoctoral Fellow
[g]Biology Division postdoctoral investigator

3.1 DISTRIBUTIVE PAIRING OF COMPOUND AUTOSOMAL CHROMOSOMES IN FEMALES OF *DROSOPHILA MELANOGASTER*

E. H. Grell

In recent years a useful series of chromosomes has been constructed in the laboratory of E. B. Lewis.[1] They are essentially "isochromosomes" for the arms of the autosomes. Normally the left and right arms of a chromosome are attached to opposite sides of the centromere. In these new chromosomes, a left or a right arm is attached to both sides of the centromere. Analogous compound chromosomes have been known for some time.

The meiotic behavior of these chromosomes has been noted to some extent;[1–3] however, it has not been acknowledged that the behavior of these chromosomes is to be expected on the basis of the "distributive pairing model of meiosis"[4] nor even that some of the observed behavior is a manifestation of "nonhomologous pairing."[5,6] Certain quantitative aspects are expected on the size-related specificity of distributive pairing.[7,8] The corollary rules for the behavior of compound chromosomes based mainly on observations of compound X chromosomes have also been published[9] and also apply to all of the compound autosomes.

It has been observed that in oocytes carrying a compound 2L [C(2L)] and a compound 2R [C(2R)], the compound chromosomes do not assort at random, but eggs are observed to carry either a C(2L) or a C(2R) but rarely both or neither (0.9%). This is expected, because both compound chromosomes are in the distributive pool where nonhomologous and homologous chromosomes can pair and be oriented to opposite poles of the first meiotic division spindle. They are in this pool along with the tiny fourth chromosomes, but they are large and similar in size, so that they pair and segregate independently of the fourth chromosomes. If a heterozygous inversion is present in the third chromosomes, the eggs that contain both or neither compound are increased (2 to 5% depending on the inversion). The reason is that the heterozygous inversion limits crossing-over, and the no-exchange third chromosomes are then in the distributive pool. The third chromosomes in the pool are then able to interfere with the pairing of the compounds and make their segregation less regular.

More direct proof of distributive pairing of compounds is made by introducing a Y chromosome into females carrying C(2L) and C(2R). The Y chromosome does not undergo crossing-over and therefore is always in the distributive pool.[4] It is close enough to the size of C(2L) and C(2R), and so it can affect their segregation rather markedly. In Y;C(2L);C(2R) females the eggs with both or neither compound are up to 28%. That is, the compounds are distributed nearly at random with respect to one another. Furthermore, the eggs that have neither compound always carry a Y, and conversely eggs with both compounds never carry a Y. The Y participates in the events that determine the segregation of the compounds.

A further clear-cut demonstration of nonrandom assortment was in females carrying a Y, a compound 2L, and two free 2R chromosomes. The two free 2R chromosomes usually cross over and are not in the distributive pool. The Y and C(2L) are the only large chromosomes in the pool and were expected to segregate rather regularly to opposite poles of the meiotic spindle, which they do in about 95% of the oocytes. The conclusion is that much of the behavior of compound autosomal arms is from the fact that they are always members of the distributive pool, and hence their segregation is dependent on the number and nature of other members of the pool. If the rules for distributive pairing are applied, the segregations can be predicted with considerable accuracy.

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3.2 MUTANTS LACKING α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY IN *D. MELANOGASTER*

E. H. Grell

The α-glycerophosphate cycle in insect flight muscle appears to be important in the rapid and efficient production of energy in that tissue. The NAD-requiring soluble α-glycerophosphate dehydrogenase is one of the enzymes in that cycle.

Ethyl methanesulfonate-induced mutations of the structural locus of α-glycerophosphate dehydrogenase (α-GDH) were selected and studied. These mutants have no detectable α-GDH activity. Homozygotes have reduced viability and are short-lived. Females have greatly reduced fertility and only rarely lay a mature egg. Males are regularly fertile. That they are at all viable was unexpected in view of the importance of this enzyme.

3.3 RESPONSE OF ALCOHOL DEHYDROGENASE LEVELS TO DOSES OF ITS STRUCTURAL GENE IN *DROSOPHILA MELANOGASTER*

E. H. Grell

The genetic and cytological position of a locus (Adh) which controls electrophoretic behavior of alcohol dehydrogenase is well known.[1] Most likely

the electrophoretic variants reflect amino acid differences, and hence the gene under study specifies the primary structure of the enzyme. Chromosomes with two rather than the normal one dose of the Adh gene were constructed. A deficiency which eliminates the Adh gene was also used in this study. In the range of from one to four doses of the Adh gene, the amount of enzyme per fly is approximately proportional to the gene dosage. Thus it appears that control of the enzyme level is dependent on gene dosage.

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3.4 THE GENETIC VARIATIONS OF *DROSOPHILA MELANOGASTER*

D. L. Lindsley
I. Lucile Norton
E. H. Grell
Elizabeth S. Von Halle

We have been working since January 1963 on a revision of "The Mutants of *Drosophila melanogaster*," published by C. B. Bridges and K. S. Brehme in 1944 and out of print for over five years. In October of 1967 the finished manuscript was at long last submitted to the Carnegie Institution of Washington for publication, and we have been assured that it will appear during the latter half of March 1968. The book contains descriptions of between 4500 and 5000 different genetic variants and is nearly complete through December of 1966. It is divided into seven sections: (1) mutants, with more than 3000 entries; (2) chromosome aberrations, including (*a*) deficiencies, (*b*) duplications, (c) inversions, (*d*) rings, (*e*) translocations, and (*f*) transpositions, with around 1600 entries; (3) special chromosomes, including (*a*) balancer chromosomes, (*b*) compound chromosomes, (c) *X-Y* combinations, and (*d*) marked *Y* chromosomes; (4) departures from diploidy; (5) cytological markers; (6) wild-type stocks; and (7) nonchromosomally inherited effects. The last five sections contain relatively few entries. In addition to this material, which occupies 430 pages, there is a list of all known or suspected loci listed in genetic order on the linkage map with indications of all known cytological positions; this list occupies an additional 40 pages. Finally, Bridges' original salivary maps and the revised maps of each chromosome arm are to be included as foldouts at the back of the book.

In the process of writing the book, each mutant has been described with respect to 15 categories of information and each chromosome aberration with respect to 20. This information has been committed to magnetic tape, and a program was written by C. E. Price of the K-25 Computer Services Division that allows the listing of information sorted with respect to one or a hierarchy of these categories of information. This possibility was extremely useful in assuring consistent usage throughout the book; it has also proven invaluable in experimental design. For example, one might like to know all rearrangements with breakpoints in a certain region or all mutants affecting electrophoretic mobility of enzymes; this information is readily extractable from the magnetic tape. It is certain that the book will be of vital importance to the field, and we anticipate that the tape will also be of continuing importance.

3.5 STUDIES OF RECOGNITION AT EXCHANGE PAIRING

Rhoda F. Grell

Pairing for exchange is generally interpreted according to the parasynaptic model, which assumes that homologous contact, initiated during the cytologically visible stages of meiosis, occurs along the entire length of the chromosome; or, less frequently, according to the "effective pairing site" model, in which contact, established prior to meiotic prophase, is limited to short discrete regions. Tests of these models have been carried out under competitive conditions provided through the incorporation of duplications into the genome. In such studies with *Aspergillus*, Pritchard[1] found that a duplication caused a large increase in crossing-over in the region of triplication, a result predictable from his "effective pairing" hypothesis. By contrast, early studies with *Drosophila* showed that duplications, although rarely involved in exchange, caused a depression in crossing-over in the triplicated region. The degree of depression, according to Dobzhansky,[2] was correlated with the total length of the duplication.

A revinvestigation of this problem has been carried out in *Drosophila*, utilizing a series of X

duplications of different total lengths and of well-defined euchromatic and heterochromatic composition. Information concerning the following points was desired:

1. the maximal reduction potential of a duplication,
2. the relationship between total duplication length and recognition,
3. the length of homology required by a duplication for full recognition (defined as recognition equivalent to that obtained for the same region carried on a normal chromosome).

The free X duplications form a series whose euchromatic content varies from 6 to 100% of the region between the tip of X and the locus of white (3C2). With respect to total length, they range from 1.1 to 3.8 times the length of chromosome 4. The two attributes are uncorrelated. The duplications were introduced, individually, into females of identical genotype whose X chromosomes were properly marked for measuring crossing-over between the tip and the locus of white, and the effect of each duplication on the frequency of exchange in this region was determined. A reduction in crossing-over of two-thirds in the designated region is considered to be equivalent to full recognition and to be a consequence of random pairing among the three elements, with exchange possible only one-third of the time, namely when the duplication remains unpaired.

Those duplications which failed to achieve a two-thirds reduction were subjected to an additional test to discriminate between the two following possibilities: (1) that recognition is equivalent for all duplications but that pairing in the region adjacent to the triplicated portion (between the termination of distal X homology and the locus of white) is independent of pairing in the triplicated region, in which case exchange in the adjacent region could account for the diminished effect, or (2) that such duplications carry insufficient homology for full recognition. For this test the size of the adjacent region was decreased by substituting the marker prune for white, the former lying ~60% of the distance from the tip to white. If the first explanation is valid, a reduction in the size of the adjacent region should reduce the probability of pairing in this region and correspondingly enhance the effect of the duplications; whereas, if the second explanation is correct, a change in the marker should not alter recognition qualities, and the percent of reduction achieved by each duplication between the tip and prune should remain the same as that between the tip and white.

The reductions in exchange between *y* and *w* effected by the different duplications are given in Fig. 3.5.1. The percent of reduction shows a good correlation with the amount of homology present in the duplication. The theoretical maximum of a

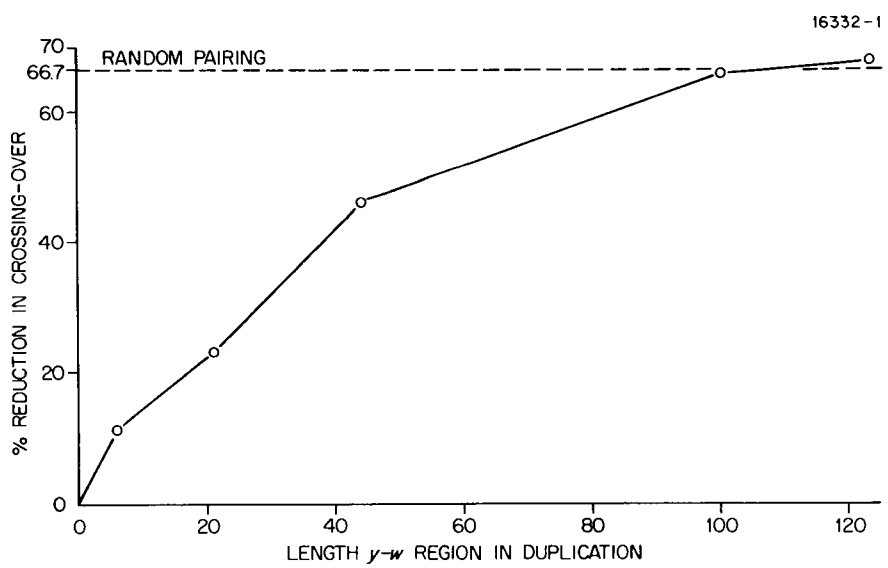

Fig. 3.5.1. Correlation Between the Extent of Homology and the Reduction in Exchange Effected by Duplications. Crossing-over measured between two normal X chromosomes in the region between *y* and *w*.

two-thirds reduction, predicted from random pairing of the two normal X's and the duplication, is reached with the two duplications which cover the region w^{m5} and z^9 (see Table 3.6.1, col. 3). A length of homology, equivalent to that lying between the tip and the locus of white, is, therefore, sufficient and may be in excess of that required for full recognition.

Contrary to the conclusion of Dobzhansky, recognition is not correlated with total duplication length. Heterochromatic regions, which comprise the remainder of the duplications' lengths, apparently do not play a competitive role in exchange pairing. Since crossing-over is extremely rare in such regions it is tempting to speculate that failure of exchange may be a reflection of failure of exchange pairing.

The three duplications which failed to attain a two-thirds reduction in exchange between the tip and white gave essentially the same results when the measurement was made between the tip and prune, thus eliminating the possibility that exchange in the region adjacent to the triplication is responsible for the lesser effect and providing support for the alternative explanation, that these duplications carry insufficient homology for full recognition. The interpretation placed on these data is that, for the system employed, 44% of the region between the tip and white does not constitute the minimal length required for full recognition, and that this length lies somewhere between 44 and 100% of the region.

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3.6 A COMPARISON OF CHROMOSOME BEHAVIOR AT EXCHANGE PAIRING AND AT DISTRIBUTIVE PAIRING

Rhoda F. Grell

Genetic investigations of meiosis in the female of *D. melanogaster* have revealed that an early exchange pairing is supplemented by a postexchange *distributive pairing*, the latter conforming in many respects to the cytological descriptions of late pairings reported by Wilson and others in a variety of organisms. Previous studies were concerned with characterizing the recognition properties of chromosomes at distributive pairing. Recently we have attempted to learn more of the attributes of exchange pairing. The present studies have utilized a series of chromosomes, each of which is active at *both* events and whose genetic behavior at the two pairings may be readily analyzed and compared.

Five X duplications were employed which varied with respect to the amount of distal X euchromatin present, the amount of proximal X heterochromatin present (or proximal 4 heterochromatin in the case of w^{m5}), and the total length. Each duplication was introduced separately into genotypically identical females of the following two different types: (1) triplo-4 females carrying a homozygous translocation between chromosomes 3 and 4 and, in addition, an extra, dominantly marked, free fourth chromosome; and (2) diplo-4 females carrying the same homozygous translocation but lacking the free 4.

The behavior of each duplication at exchange pairing was reflected in its ability to reduce exchange between the two normal X chromosomes in the region between the tip, marked with yellow, and the locus of white (~1.5 crossover units), in which region the different duplications possess varying degrees of homology. A reduction of $\frac{2}{3}$ indicates random pairing between the two normal X chromosomes and the duplication and is assumed to require close to 100% participation of the duplication in exchange pairing. The behavior of each duplication at distributive pairing was measured by the frequency that it segregated from the free fourth chromosome in the triplo-4 females. The extent that distributive pairing might interfere with exchange pairing was assessed in two different ways: (1) by determining the frequencies that the same duplication may be involved in each of the two pairing processes and (2) by comparing the reduction in exchange effected by an X duplication when it is engaged (triplo-4 females) and when it is not engaged (diplo-4 females) in distributive pairing with chromosome 4.

In Table 3.6.1, the results obtained for exchange pairing are presented at the left and those for distributive pairing at the right. The extent that each duplication depresses crossing-over between the normal X chromosomes in the region from *y* to

Table 3.6.1. Behavior of Free X Duplications at Exchange Pairing and at Distributive Pairing, the Former Measured in Females $y^2/yw/Dp$; T(3;4)86D/T(3;4)86D With and Without a Free Fourth Chromosome, the Latter in the Triplo-4 Females Only

Exchange Pairing					Distributive Pairing			
Dp.	Total Length (chromosome 4 = 1)	$y - w$ Region (%)	Amount of Crossing-over $y - w$ Region		Dp.	Total Length (chromosome 4 = 1)	Duplication-4 Nondisjunction (%)	Duplication-4 Association (%)
			Diplo-4	Triplo-4				
None			1.57 ± 0.12					
1144	1.1	6	1.41 ± 0.26	1.41 ± 0.25	1144	1.1	0.13(0.02, 0.10)[a]	99.7
856	3.0	21	1.27 ± 0.18	1.14 ± 0.18	1337	1.4	0.38(0.38)[a]	99.2
1337	1.4	44	0.90 ± 0.13	0.80 ± 0.14	w^{m5}[b]	1.9	0.45	99.1
w^{m5}	1.9	100	0.54 ± 0.14		856	3.0	1.70(1.90)[a]	96.6
z^9	3.8	123	0.52 ± 0.13	0.51 ± 0.14	z^9 3	3.8	3.03	93.9

[a]Values obtained in previous experiments.
[b]Measured in females $y^2/y^2/w^{m5}$; T(3;4)86D/T(3;4)86D/ci^D.

w in diplo-4 and in triplo-4 females (columns 4 and 5 respectively) is seen to be positively correlated with the amount of homology that each duplication bears for the tip of X (column 3) and is further seen to be unrelated to the total length of the duplication (column 2). The frequencies of nonhomologous association between each duplication and the free fourth chromosome [as calculated from nondisjunction values (column 8) by means of the expression: association = 1 − 2 × nondisjunction] are given in column 9 and are seen to be positively correlated with total duplication length (column 7). The inefficacy of homology in the latter process is demonstrated by the behavior of w^{m5}, which assumes a position in the series (column 9) that is consistent with its total length, despite its shared homology with the base of chromosome 4.

Recognition at the two pairings is, therefore, dependent on different attributes of the same duplication. For exchange pairing, homology is required, the degree of recognition is correlated with the extent of homology, and total size is irrelevant. For distributive pairing, recognition is correlated with total size, and homology is not a factor. Is distributive pairing competitive with exchange pairing? Estimates of the frequencies that a single duplication may be involved in homologous exchange pairing (approaching 100% for w^{m5} and z^9) and nonhomologous distributive pairing (94 to 99%), particularly in view of the distinct recognition properties of each, provide strong evidence for the discreteness of the two processes. This conclusion finds further support in the observation that the participation of a duplication in distributive pairing fails to alter its effect on exchange. Thus the depression in exchange induced by each duplication in triplo-4 flies (column 5) is at least as great in every instance as that induced by the same duplication in diplo-4 flies (column 4), notwithstanding the fact that the duplications are highly involved in distributive pairing in the former case but not in the latter. The two independent lines of evidence cited above provide additional, compelling evidence for the temporal separation of the two pairing processes.

3.7 DNA REPLICATION AND EXCHANGE IN THE *DROSOPHILA* OOCYTE

Rhoda F. Grell J. W. Day

Temperature treatment and labeling studies indicate an approximate coincidence between the temperature-sensitive period for crossing-over and that of DNA replication in the oocyte of *D. melanogaster*.[1] For a more precise temporal resolution of the two periods, investigations have

been carried out utilizing immature females from which it is possible to subsequently recover a well-synchronized population of eggs representing predominantly the first oocytes to undergo maturation. Correlated cytological and genetic studies have shown that the initiation of the heat-sensitive period corresponds well to the initial appearance of the oocyte in the pupa (~126 hr after egg laying) and that the maximal heat-induced increase in exchange corresponds to the attainment of a steady-state population of oocytes (~144 hr).[2] Studies are now in progress with a view to answering the following questions:

1. Do different chromosomal regions respond synchronously to heat treatment? If not, does a pattern emerge?
2. How soon after oocyte formation does DNA replication begin?

To investigate the possibility of an asynchronous response, four suitably marked contiguous regions on chromosome 2 have been studied (Fig. 3.7.1). Groups of developing females, differing in age by 6 hr and representing ages between 120 and 168 hr, were subjected to a heat treatment of 35°C ± 0.5 for 12 hr. The frequencies of exchange within each of the four regions at each of the 6-hr intervals were measured.

To ascertain the time of DNA synthesis in the pupal oocyte, ovaries taken from pupae between 132 and 168 hr old were incubated in ^{3}H-thymidine, and autoradiographs were prepared and examined for the presence of label in the most posterior cysts of the ovarioles within which the oldest oocytes lie.

Quantitatively different responses were observed for the four regions, which correspond to the proximity of the region to the centromere (Fig. 3.7.1), so that region 4, the most distal, shows the least response and region 2, the most proximal, the greatest response. Initiations of the responses were found to be approximately synchronous, but the terminations varied, with region 4 responding only up to 150 hr, region 3 until 162 hr, and regions 1 and 2 until 168 hr. Region 2 reached its maximal response at a time (156 hr) when region 4 had been at a control level for at least 6 hr. It should be

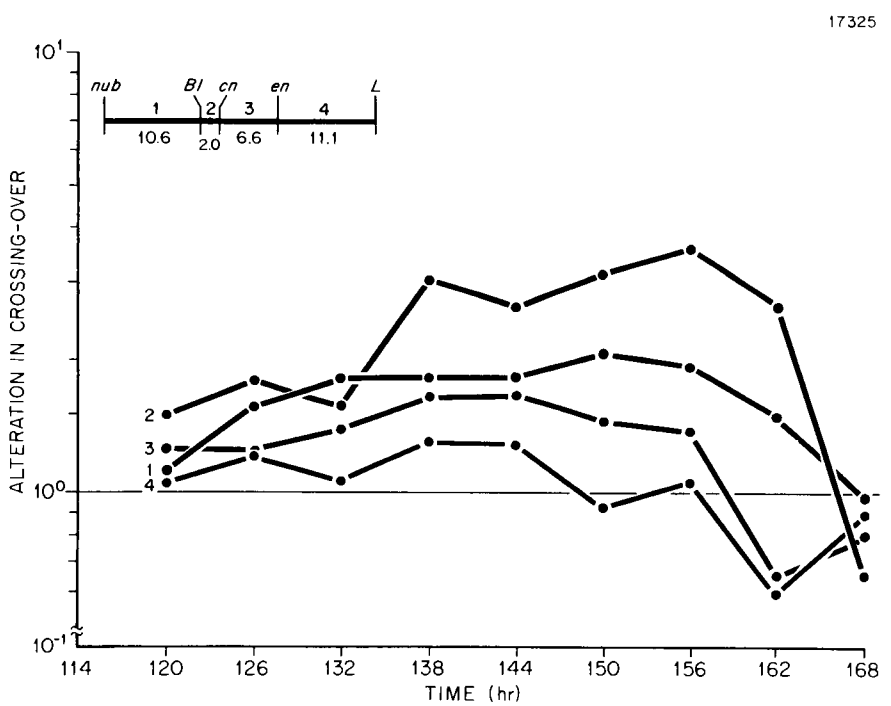

Fig. 3.7.1. The Responses of Four Regions on Chromosome 2 to 12-hr Temperature Treatments Initiated at 6-hr Intervals from 120 to 168 hr.

of interest to determine whether corresponding regions of other chromosomes display a similar pattern.

Autoradiographs of the pupal ovaries showed label to be present in the most posterior 16-cell cysts of the ovarioles between 138 and 156 hr. The period of DNA synthesis, therefore, shows a good correspondence to the period of maximal temperature response (138 to 156 hr). These results confirm and extend our earlier observations with adult females. They indicate that the temperature-sensitive period is at least partially coextensive with the period of DNA synthesis in the oocyte.

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3.8 NONHOMOLOGOUS PAIRING IN THE OOGONIA OF *D. MELANOGASTER*

Rhoda F. Grell J. W. Day

Earlier cytological studies demonstrated that nonhomologous chromosomes undergo a size-dependent pairing in the oogonial cells of *D. melanogaster* during the division stages. To compare the intimacy of such pairing with that which normally occurs between homologs in the same cells, photographic enlargements of a large sample of such cells were studied. Using a binocular fitted with an 8× ocular micrometer, the distances between the members of each homologous pair and the distance between the two nonhomologs were measured. The distance between nonhomologs divided by the average distance between homologs for a particular cell yielded a quotient with a value of 1 if the distances were alike, less than 1 if the nonhomologs were more closely paired, and greater than 1 if the homologs were more closely paired.

For nonhomologs previously classified as paired, the average quotients for the five different genotypes that were studied were 0.9, 1.0, 1.0, 1.1, and 1.2. For nonhomologs previously classified as unpaired, the average quotients for the same five genotypes were 2.7, 2.8, 2.9, 3.2, and 3.5. Judging by the criterion of distance, the intimacy of nonhomologous pairing, when it occurs, is approximately as great as the intimacy of homologous pairing in the same cell.

3.9 DEMONSTRATION OF FEMALE FERTILITY OF HOMOZYGOUS MALE-STERILE *X*-AUTOSOME TRANSLOCATIONS

D. L. Lindsley Mary S. Uziel

It is known that a large fraction of translocations between the *X* and either the second or the third chromosome in *Drosophila melanogaster* are male sterile. They do not affect the fertility of heterozygous females, however. Until now it has not been possible to produce translocation homozygotes because of the necessity for both the father and the mother to contribute the male-sterile translocation to such a homozygous offspring. The finding that the meiotic mutant *mei-S332a* produces a high frequency of nondisjunction in the second meiotic division presented us with the capacity of producing ootids that were simultaneously disomic for both elements of a male sterile *T(X;A)*. Fertilization of such a doubly disomic egg with a *Y*-bearing sperm nullosomic for the autosome involved in the translocation should result in the production of a viable female homozygous for the *T(X;A)*; such sperm are produced regularly by males carrying appropriate compound autosomal arms. Since *mei-S332a* is on the second chromosome, two male-sterile *T(1;3)*'s were chosen, *T(1;3)SP37* = *T(1;3)8F;64E* and *T(1;3)SP53* = *T(1;3)12;92*, and made homozygous for *mei-S332a*. *T(1;3), y w/FM6; mei-S332a* females were then crossed to *C(3L)RM, se* h^2 rs^2; *C(3R)RM, sbd gl* e^s males. Several *y w* females, which were presumably *T(1;3), y w/T(1;3), y w; mei-S332a/*± were recovered; these were outcrossed and produced *y w* sons, all of which were sterile. This recovery of sons confirms that the *y w* females recovered were fertile, and the production of all *y w* sterile sons shows that the females were homozygous for the translocation. This exercise demonstrates how meiotic mutants can serve as useful tools in problems unrelated to the study of the meiotic mutants themselves.

3.10 AUTOSOMAL NONDISJUNCTION IN MEIOTIC MUTANTS OF *DROSOPHILA MELANOGASTER*

D. L. Lindsley Mary S. Uziel

Three mutations that increase the incidence of nondisjunction of the *X* and the fourth chromosomes were extracted from samples of natural populations of *Drosophila melanogaster* collected near Rome, Italy. We examined the effects of these mutants on nondisjunction of the large autosomes, chromosomes *2* and *3*. This was done by crossing *y/y; mei-/mei-* females with males carrying an *X* chromosome marked with y^+ and compound autosomal arms [either *C(2L)RM; C(2R)RM* or *C(3L)RM; C(3R)RM*]. The two compound chromosomes remain univalent in the primary spermatocyte; consequently, sperm are formed with neither, one, the other, or both compound chromosomes in roughly equal frequencies, as shown in Fig. 3.10.1. It can also be seen in Fig. 3.10.1 that the only surviving progeny from a cross of compound-autosome-bearing males and females carrying normal autosomes are derived from meiocytes in both parents in which the two autosomal elements proceed to the same pole at MI or MII. Since only exceptional products of oogenesis are recoverable from this cross it is possible to determine that autosomal nondisjunction is occurring, but it is not possible accurately to estimate its frequency. It is further possible to examine the incidence of *X*-chromosome exceptions in a sample of autosomal exceptions obtained from this cross for comparison with that observed in crosses using normal autosomes where autosomal disjunction is regular. It is also possible to examine the incidence of *X*-chromosome exceptions among fourth-chromosome exceptions in crosses to males carrying *C(4)RM*. The results are presented in Table 3.10.1. It can be seen that a rather large number of autosomal exceptions was recovered from each mutant type; comparable numbers of control matings would have yielded fewer than ten autosomal exceptions.

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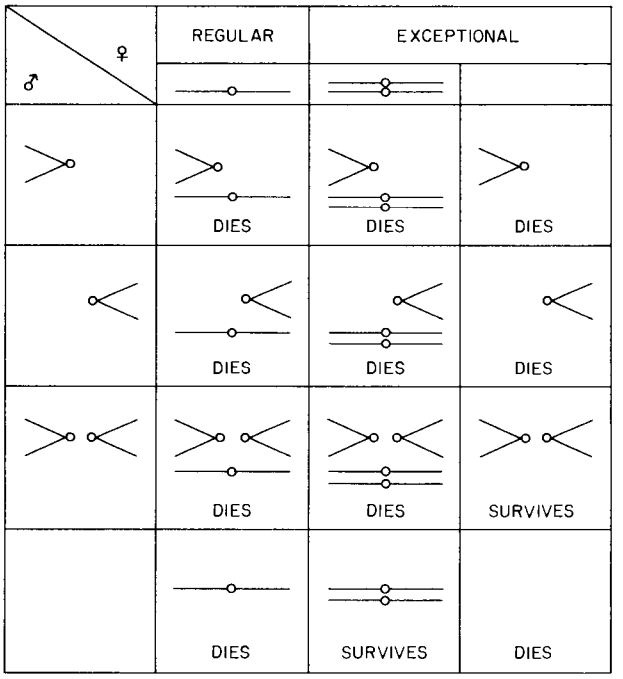

Fig. 3.10.1. Gametic and Zygotic Genotypes Produced in Cross of *C(2L)RM; C(2R)RM* or *C(3L)RM; C(3R)RM* Males with Normal Females.

Among autosomal exceptions produced by *mei-S51* there were 47% (133/284) *X*-chromosome exceptions compared with 1% (12/1058) from oocytes in which autosomal disjunction was regular. Thus, disjunctional behavior of the *X*'s and the autosomes within a cell is strongly correlated. Furthermore, among simultaneous *X* and autosomal exceptions the two *X* chromosomes separate from the two autosomes 90% (119/133) of the time. These observations suggest that in a small fraction of the oocytes of *mei-S51* females, pairs of chromosomes pair nonhomologously and separate from each other at the first meiotic division. This is correlated with a reduction of recombination in the *X* chromosome and presumably in the autosomes to about half the normal value. Such negative correlations between exchange frequency and nonhomologous pairing have been shown for a large number of different genetic situations by R. F. Grell [see *J. Cell Physiol.* **70** Suppl. 1, 119 (1967)].

The autosomal exceptions recovered from *mei-S282* females also show a highly correlated recovery of *X*-chromosome exceptions, that is, approximately 30% (14/49), compared with less than 1% (10/1529) among regular autosomal segregants. Among the double exceptions, the two *X*'s went to the same pole as the two autosomes (six times) and to the opposite pole from the two autosomes (eight times) with equal frequency. The results agree with expectations based on random recovery of *X* chromosomes among

Table 3.10.1. *X*-Chromosome Genotype of Autosomal Exceptions Produced by Meiotic Mutants

Mother	Genotype of — Primary Oocyte with Respect to — *X* Chromosome	Autosomes 22	0	33	0	44	0	ΣAA	Σ0
mei-S51	*X*	49	41	28	25	6	2	83	68
	XX	4	24	3	12	1	1	8	37
	0	38	2	37	4	7	0	82	6
mei-S282	*X*	1	3	12	14	2	3	15	20
	XX	0	0	4	1	1	0	5	1
	0	0	0	5	0	2	1	7	1
mei-S332a	*X*	2	4	43	18	17	28	62	50
	XX	0	0	2	1	2	3	4	4
	0	1	3	2	17	2	10	5	30

autosomal exceptions and suggest that in a small fraction of cells there is complete failure of synapsis, followed by random segregation.

For *mei-S332a* females the frequency of *X*-chromosome exceptions among autosomal exceptions (43/155 = 28%) is approximately the same as among regular autosomal segregants (23/105 = 22%). Thus in *mei-S332a*, which is known to interfere with the second meiotic division, the *X* chromosomes and the autosomes appear to behave independently. Among simultaneous *X*-chromosome and autosomal exceptions, however, there is a great excess of gametes that receive neither an *X* nor an autosome, suggesting some type of interaction that is not readily explainable.

3.11 DNA MEASUREMENTS ON HETEROCHROMATIN

J. R. Merriam Stella W. Perdue[1]
R. F. Kimball[1]

Heterochromatin accounts for about one-third of the mitotic chromosomes but less than 5% of the salivary chromosomes. Therefore it is interesting to know if DNA presents a similar mitotic picture or if, as Cooper has suggested, the mitotic picture is an artifact of acetocarmine staining. Rudkin,[2] using uv microphotometry on neural ganglion metaphase chromosomes spread with isocitrate solution, has determined that approximately 25% of the *X* chromosome DNA is deleted in the $In(1)sc^{4L}sc^{8R}$ chromosome, which removes most of the *X* heterochromatin. The present report describes measurements with the scanning microspectrophotometer in Feulgen-stained testis squashes from males with an attached *XY* chromosome but no free *Y*. The conclusion is the same as that of Rudkin, that heterochromatin contains as much DNA per unit metaphase length as euchromatin does.

Table 3.11.1 summarizes all of the total nucleus measurements made on testis and neural ganglion material. There is very good agreement between the heavily condensed mitotic metaphase chromosome sets and the less heavily condensed meiotic prophase sets. However, measurements on individual sperm heads are not so fortunate, as the variation is immense but without evidence of the bimodal distribution expected, although the range falls about values expected for the haploid genome. Table 3.11.2 lists the individual measurements of those cells in which the two-strand attached *XY* chromosome could be cleanly distinguished from the four-strand autosomes. There is again good agreement within and between tissues, and the overall average of 5.2 units for the *XY* chromosome is a reasonable value to compare against the replicated genome average of 23 to 24 units. Thus, each single strand of the attached *XY*, with only

Table 3.11.1. The Results of Measurements with the Scanning Microdensitometer in Integrated Absorbance Units on Feulgen-Stained Nuclei from Testis and Brain Squashes of $Y\hat{S}X \cdot Y\hat{L}$, *In(1)EN, y B/O* Males

All measurements are from a single slide on which two cysts of meiotic prophase I cells in a testis squash were identified cytologically and on which a 3d larval instar brain from a male of the same stock was included for comparison

Tissue	Class	Number Measured	Average	Range
Testis	Prophase I	21	22.9	16.8–26.5
Brain	Metaphase	15	23.3	20.5–27.2
Brain	Diffuse { 2c ? / 4c ?	12 / 3	~12 / ~24 }	10.3–27.1
Brain	Telophase (segregating nuclei)	12	~11	8.4–12.7
Sperm heads		47	Median = 5.5	3.3–9.6

Table 3.11.2. Measurements on Individual $\overline{XY}$ Chromosomes in Testis and Brain Cells

Cell No.	Testis Prophases		Cell No.	Brain Metaphases	
	$\overline{XY}$	Total Cell		$\overline{XY}$	Total Cell
1	6.2	26.5	1	4.0	21.3
2	4.6	23.7	2	4.5	23.7
3	4.5	23.9	3	5.4	23.4
4	5.4	25.9			
5	6.2	22.4			
6	6.1	25.3			

one euchromatic arm, contains as much Feulgen-staining material as does each single-strand autosome with two euchromatic arms (2.6 units vs $(23.5–5.2)/8 \cong 2.4$ units). Therefore we can conclude that the Y-heterochromatic arm contains as much DNA as the X-euchromatic arm does.

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3.12 CHROMOSOME PAIRING AND FERTILIZATION IN MALE *DROSOPHILA MELANOGASTER*

J. R. Merriam

It is well known that in XYY males more than 67% of the sperm recovered are Y- or $(X + Y)$-bearing, while both Y's together or the X alone are rarely recovered. This is usually interpreted to mean that conjugation, during meiosis and subsequent disjunction, of the three elements is nonrandom, which implies a specific mechanism of chromosome pairing. Although it is not possible to obtain pairing frequencies directly with marked chromosomes, there being at least four equations with only three observations, it should still be possible to define some of the experimental parameters which influence the implied mechanism. One such parameter, for example, is whether the number of *arms* or the number of centromeres determines the pairing relationships. It can be seen in Table 3.12.1 that crosses with $FM1/B^SY/sc^{V1} \cdot YS$ males ($sc^{V1} \cdot YS$ is one-armed) yield results that are not significantly different from the typical results of control crosses with half brothers of the constitution $FM1/B^SY/y^+Y$. Thus it would seem that reducing the number of arms from 5 to 4 does not significantly alter conjugation. In the

Table 3.12.1. Results of Crosses of *XYY* Males with Sex Chromosomes Marked with *y*, B^S, and y^+ to *y cv v f* Females

Male Constitution	Progeny Classes[a]					
	y ♀♀	y^+ ♀♀	*y* B^S ♀♀	y^+ ♂♂	*y* B^S ♂♂	y^+ B^S ♂♂
$FM1/sc^{V1} \cdot YS/B^SY$	154	547	767	621	573	140
$FM1/y^+Y/B^SY$	62	219	246	213	195	31
$X^{c1}/y^+Y/B^SY$	524	552	627	608	649	311
$y/Dp3/B^SY$	852	607	248	540	594	334

[a]Males are *cv v f* as well.

same experiment, in crosses of brothers bearing the ring *R(1)1* (marked with *y*) the distribution of chromosomes recovered among sperm changes to one suggesting random 2 : 1 disjunction. This has later been repeated with certain rod chromosomes. However, crosses with $y/B^SY/Dp(1;f)3$ males [*Dp(1;f)3* = one-armed *X* heterochromatin] yield results (Table 3.12.1 and repeated) so completely different that the initial assumption that recoveries of chromosomes among sperm reflect meiotic patterns is no longer justified. In the first place, whether the selection is meiotic or post-meiotic, it is clear that the one-armed *Dp(1;f)3* is not equivalent to the one-armed $sc^{V1} \cdot YS$ (although they are of equal size), and therefore it is not chromosome construction but *content* which is important here, in contrast to the meiotic situation in females, where construction (size, etc.) is important. In the second place, complementary gamete classes are *not* recovered in equal frequency, and the total recovery of each element is not 50%, although on any meiotic segregation model such results are clearly not expected. The serious possibility exists that the apparent nonrandom recovery of chromosomes is an artifact of some post-meiotic sperm selection, and therefore has nothing to do with meiosis. Regular sperm selection by females has been demonstrated in *Drosophila* (Olivieri). Thus future genetical experimentation on male meiosis must first delve into the physiology and phenomenology of sperm transfer, storage, and fertility in order to establish the necessary first principles. In turn, the genetic results with marked chromosomes described here should provide a convenient tool for investigating the events leading to fertilization.

3.13 ON THE SEQUENCE OF ELEMENTS IN COMPOUND-*XY* NO. 129-16 AND IN SOME OF ITS DERIVATIVE MARKED *Y* CHROMOSOMES

D. R. Parker

A number of detachments resulting from induced exchange between an attached *X* and the y^+Y were found to be attached-*XY*, and were considered to be of the sequence $XY^S \cdot Y^Ly^+$ if the marker was retained (Parker and McCrone, 1958). One stock, No. 129-16, that has been used in making some doubly marked *Y*'s has been found instead to be of the sequence $Xy^+Y^L \cdot Y^S$, with the break in the y^+Y distal to ac^+ and y^+, but proximal to $1(1)J1^+$. This stock differs from the other compound-*XY* detachments that retain y^+ in having some yellow variegation in combination with yellow or yellow^2 in the attached-*XY/O*. Variegation in the presence of yellow^2 is most easily seen as pale spots in the darker sclerites at the tip of the male abdomen.

One derived *Y* (*Y-66d*, $Y^Sy{+}Y^L \cdot Y^S$) arose spontaneously in a *129-16/O* male and was recovered as a single *C(1)RM, y v bb/Y-66d* female. Males of the constitution *y sc/Y-66d* are fertile and show a more pronounced yellow variegation than *y sc 129-16/O* males do. Variegation in this case and in the case of the original *129-16* stock suggested a different relationship of the yellow locus in these stocks. Furthermore, were y^+ in a terminal rather than in an interstitial position, the derived *Y*, having two doses of the y^+ duplication, should show the pronounced *Hw*-like phenotype of extra hairs in the second posterior cell of the wing, branched posterior crossveins, etc., that is

regularly found in iso-marked y^+Yy^+ chromosomes (Williamson).

A test of the position of the y^+ marker was to reconstitute an attached *X* from *129-16*. Were the marker terminal, it should almost surely be lost in forming a new attached *X*; if interstitial, it should be retained. One such attached *X* arose spontaneously in the *129-16/O/C(1)RM, y v bb* stock; it retained the marker. The interstitial position was further confirmed by finding that the y^+ duplication in *129-16* and in *Y-66d* did not cover *1(1)J1* either with or without an extra *Y*.

Williamson has obtained a number of spontaneous attached *X* detachments from a doubly marked *Y* derived from *129-16*, where the detachments carried either both of the markers (y^+ and B^S) or neither, showing this derivative to be B^Sy^+Y rather than y^+YB^S as formerly believed.

The interstitial position of y^+ in *129-16* suggested that *Y-66d* could carry two doses of the Y^S male-fertility complex (*KS*) as well as one of the Y^L complex (*KL*), hence should be of the structure $KS\ y^+\ KL \cdot KS$. This was verified by testing a series of radiation-induced detachments, using ability to complement $y^+ \cdot Y^L$ as a test for the presence of a complete *KS*. Of eight detachments that carried y^+, and therefore should in any simple exchange also carry one or the other of the *KS* complexes, seven were found to give fertile males in combination with *FR-2*. The eighth case proved to be a complex one, having all of the markers of *4R* as well as y^+ and *k1-5* linked with the *X*, requiring a total of four breaks to form the detachment.

3.14 SOME DISJUNCTIONAL CONSEQUENCES OF TRANSLOCATION INDUCTION IN *DROSOPHILA* OOCYTES

D. R. Parker

One may ask why the rarity of recovery of reciprocal translocations following irradiation of the female? The possibilities have been eliminated that it is due to a low breakability of the chromosomes or to failure to rejoin. It may or may not be due to some special geometry of the nucleus that gives preference to interhomolog exchange. Observations have been largely limited to the recovery of "induced crossovers" or "pseudocrossovers" or to "half translocations." Vast differences in the expected recoverability (due to aneuploidy) of various kinds of those products whose occurrence one can postulate suggest that observations may be strongly biased by the screening procedures.

The possibility suggested itself that there is some direct relationship between interchange induction and disjunction, such that elements of the newly arisen translocation are directed to opposite poles at anaphase I. A "pseudobivalent" would be formed by the interchange and might behave disjunctionally as do true bivalents tied together by a chiasma.

Two types of experiments have been tried. One involves chromosomes not normally expected to form meiotic bivalents, where two centric elements are held together by chiasmata (detachments of attached-*X/O* by exchange with chromosome *4*); the other involves whole-arm translocations between chromosomes *2* and *3*, where homologs usually would be expected to have undergone crossing-over, hence to present a more complex disjunctional problem.

The principle of the detachment experiments is that the analysis of the detachment classes found to be hyperploid for a part of chromosome *4* should reveal the division at which the two halves of the rearrangement separated. If disjunction is at division I, the maternally derived fourth chromosome elements should be from homologs; if division II, they should be sisters. A model of directed disjunction requires division I disjunction. The technique has been that of differential marking of the two fourth chromosomes, followed by appropriate testing of the recovered detachments to determine the maternal contribution to the detachment-carrying flies. Two different systems have been used; the first employed $ci^D/Dp(1;4),\ y^+$, the second $spa^{pol}/Dp(1;4),\ y^+$. The results are given in Table 3.14.1. It can be seen that there is recovery of the homolog of the broken fourth in 20/22 cases (or 23/25 if one grants that the three detachments lacking chromosome *4* markers do indeed have an unmarked *4L* capping the detachment). One of the two exceptions is seen to have a recombinant free fourth chromosome, part sister, part homolog. Since we find a fairly high frequency of three- and four-break events, involving three or more elements, one would expect exceptions of the kind where two of the three elements proceed to one pole at AI, the other to the opposite pole. Sometimes the disjunction at AII might lead to the recovery of an unbroken element, hence no evidence for the occurrence of multiple breakage. It can be said, how-

Table 3.14.1. Analysis of Hyperploid Detachment Classes

Experiment No.	Maternal Chromosomes Contributed – $X{\cdot}4L,\ y^+/ci^D$ or $X{\cdot}4L,\ y^+/spa^{pol}$	$X{\cdot}4L\ ?/y^+{\cdot}ci^+\ spa^+$	$X{\cdot}4R,\ ci^+\ spa^+/spa^{pol}$ or $X{\cdot}4R,\ ci^+\ spa^+/ci^D$	$X{\cdot}4R,\ ci^D/y^+{\cdot}ci^+\ spa^+$ or $X{\cdot}4R,\ spa^{pol}/y^+{\cdot}ci^+\ spa^+$	Exceptions
1	3	(2)[a]	2	5	1[b]
2	5	(1)[a]	3	2	1[c]
Total	8	(3)	5	7	2

[a]The presumption is that most detachments not carrying a linked chromosome *4* marker are capped by the unmarked left arm of the fourth chromosome marked with ci^D or with spa^{pol} in the right arm.

[b]$X{\cdot}4R,\ ci^+\ spa^+/y^+{\cdot}ci^+\ spa^+$.

[c]$X{\cdot}ci^+\ spa^+/ci^+\ spa^+$.

ever, that following simple two-break exchanges between attached *X* and *4*, most if not all of the time the two halves of the translocation proceed to opposite poles at the first division.

Another experiment involved irradiating females homozygous for four markers, one in each arm of each major autosome. These females were crossed to males heterozygous for a whole-arm *T(2;3)*, with the expectation that aneuploid segregation following the induction of a new *T(2;3)* might be balanced by the aneuploidy of sperm resulting from one kind of adjacent disjunction. This should be recognizable by homozygosis in the F_1 of one of the markers used.

Backcrossing such flies to the homozygous recessive parental stock should now show them to be heterozygous for two of the markers, homozygous for the normal allele of the remaining one, and should further show that the two heterozygous markers behave as if in repulsion. This reveals how the arms of *2* and *3* have been rejoined, *2L* with *3L*, etc. A trial run was made with this experimental design, brooding the treated females over a period of several days. Three such "half translocations" were recovered; because of the small numbers and brooding techniques, estimation of rates would be meaningless, but crude estimates suggest that the frequency of this kind of interchange probably differs by much less than an order of magnitude from the frequency of detachment of attached *X* by exchange with the *Y*.

3.15 RECOVERY OF MULTIPLE-BREAK REARRANGEMENTS FROM IRRADIATED OOCYTES

J. H. Williamson D. R. Parker

Until recently it has been tacitly assumed that only simple two-break rearrangements can be recovered in oocytes of irradiated attached-*X* females. This report describes four cases of rearrangements involving three or four breaks each. In two cases, both a detachment and a *Y* fragment were recovered in sons of irradiated *C(1)RM, y v bb/*$B^S Y y^+$ females. Both detachments carried B^S and none of the *Y* fertility factors, while both *Y* fragments retained the y^+ marker and were capped by *4R*. Fertility tests showed that one fragment was a complete *Y* and that the other was broken between *kl-2* and *kl-3*. Thus in one case a minimum of three breaks must have occurred, while in the other a minimum of four breaks was necessary to produce both the detachment and the fragment.

The third case was a detachment recovered from irradiated *C(1)RM, y v bb/*$Y^S y^+ Y^L \cdot Y^S$ females that proved to contain *kl-5*, y^+, and ci^+ ey^+ spa^+. Recovered along with this was the intact $Y^S y^+ Y^L \cdot Y^S$ chromosome. The fourth example came from irradiated *C(1)RM, y v bb/O* females, where the fourth chromosomes were differentially marked ($spa^{pol}/Dp(1;4)y^+$). Recovered was a detachment capped by *4R* (spa^+), along with a free maternally derived recombinant fourth chromosome marked with spa^+ but not y^+. Thus, there were recovered products of two separate two-break events.

3.16 INTERCHROMOSOMAL EFFECTS ON DETACHMENT OF ATTACHED-*X* CHROMOSOMES IN *D. MELANOGASTER*

J. H. Williamson

In *Drosophila* females, autosomal inversion heterozygosity increases recombination in the *X* chromosomes but does not affect the frequency of spontaneous detachment of attached-*X* chromosomes. However, Lucchesi[1] reported that the frequency of *x*-ray-induced detachment of attached *X*'s was higher in females with one autosomal inversion than in females with no inversion and higher yet in females with an inversion in each of the two large autosomes. He argues that the induction of detachments is influenced by homology between the *X* and the *Y* and that the increased frequency of detachments in the presence of autosomal inversions is not different from increased recombination in normal *X*'s in the presence of autosomal inversions. If this is the case, then the increase in frequency of *X-Y* detachments should occur at the expense of *X-4* detachments.

Lucchesi's experiment was repeated with slight modifications which allowed recognition, recovery, and analysis of all induced exchanges, as well as the substitution of *In(3LR)*Ubx^{130} for *In(3LR)CxD*. The data are presented in Table 3.16.1. In the presence of *SM5* and both *SM5* and Ubx^{130} the frequency of *X-Y* detachments is significantly higher than the control value. However, in the presence of Ubx^{130} alone the frequency of *X-Y* detachments is significantly lower than the control frequency, as is the frequency of *Y* fragments. In no case did the frequency of *X-4* detachments differ. While both *In(3LR)CxD* and *In(3LR)*Ubx^{130} markedly

Table 3.16.1. Recovery of X-Ray-Induced Detachments and *Y* Chromosome Fragments in the Progeny of Irradiated *C(1)RM, y v bb/B^S Y y^+* Females

Genotype of Females	Number of Progeny	Detachments		*Y* Fragments	Number of Exceptional Progeny
		X-Y	*X-4*		
+/+; +/+	8217	179	48	172	357
%		2.18	0.58	2.08	4.34
+/*SM5*; +/+	4642	134	27	80	200
%		2.89[a]	0.58	1.72	4.31
+/+; +/*Ubx*^{130}	6024	96	29	98	264
%		1.59[a]	0.48	1.63[a]	4.38
+/*SM5*; +/*Ubx*^{130}	1597	54	7	29	89
%		3.38[b]	0.44	1.82	5.57[a]

[a]*P* less than 0.05.
[b]*P* less than 0.01.

increase recombination in normal *X* chromosomes, they obviously differ in their effects on induced detachment of attached-*X* chromosomes. This difference may be interpreted as supporting the contention that detachment of attached *X*'s is not the same as classical crossing-over. At any rate, other inversions should be tested and their effects on detachment and crossing-over compared.

Reference

[1]J. C. Lucchesi, *Genetics* **54**, 1013–18 (1966).

3.17 IDENTIFICATION OF *Y* FRAGMENTS WITH TWO DOSES OF y^+ OR B^S

J. H. Williamson

Parker[1] has suggested that nonreciprocal exchanges between the arms of the *Y* chromosome may account for a large proportion of *Y* fragments induced in irradiated females. When the irradiated *Y* chromosome is the $B^S Y y^+$, nonreciprocal exchanges between the two arms would produce fragments with two doses of y^+ or two doses of B^S. Flies carrying two y^+Y's have extra hairs in the second posterior wing cell (Schultz, via Lindsley). This suggested to Parker (personal communication) that *Y* fragments with two doses of y^+ may cause extra hairs in the wings of flies carrying the fragment. Of 190 y^+ fragments recovered in males, 67 (35.3%) carried *4R* and 81 (42.6%) caused extra hairs in the wings of flies carrying the fragments. Not only did the wings possess extra hairs but there were also extra vein cells in the wings, especially associated with the posterior cross vein. These abnormalities are probably due to variegation of the Hw^+ locus in the sc^8 duplication, two doses of which are necessary for expression. In a sample of 56 B^S fragments, 29 (51.8%) produced smaller eyes than one dose of B^S did.

The real proof of isomarked *Y* fragments is to make detachments of attached *X*'s using these fragments and to analyze all of the detachments for y^+ or B^S and *4R*. This has been done, and over 90% of the detachments made with presumptive isomarked fragments have been shown to be *X-Y* or *X-4R* detachments, while less than 70% of the detachments made with $?Yy^+$ or $B^SY?$ fragments were so identified. Thus approximately 80% of the y^+ fragments and 50% of the B^S fragments have been identified, allowing inferences as to the types of induced exchanges producing *Y* fragments.

Reference

[1]D. R. Parker, *Mutation Res.* **2**(6), 523–29 (1965).

3.18 EMS-INDUCED STERILE *Y* CHROMOSOMES IN *DROSOPHILIA MELANOGASTER*

J. H. Williamson

Young *Drosophila melanogaster* males carrying a doubly marked *Y*, B^SYy^+, were fed ethyl methanesulfonate and mated in mass to attached-*XY*, *1(1)J1 y w/In(1)FM6*, y^{31d} sc^8 *dm B* females. Male progeny of the genotype attached-*XY*, *1(1)J1 y w*/$B^SY{*}y^+$ were mated individually to *C(1)TA*, *v/O* females. (The y^+ duplication on the *Y* carries the normal allele for *1(1)J1* and thus covers the lethal in the *X* chromosome. The asterisk indicates a treated *Y*. *C(1)TA* is a tandem acrocentric compound *X* chromosome in which crossing-over generates single rod *X*'s.) The progeny of such crosses were *C(1)TA*/$B^SY{*}y^+$ and *XY/X* females and *X*/$B^SY{*}y^+$ males. The regular males died because of the uncovered lethal. The progeny of each cross was transferred to new vials. If the *X*/$B^SY{*}y^+$ males were sterile, no larvae were observed in that vial, and the $B^SY{*}y^+$ was extracted from the *C(1)TA*/$B^SY{*}y^+$ females, put into stocks, and subsequently retested for sterility. Of 625 tested $B^SY{*}y^+$'s, 13 were shown to be sterile *Y*'s. These sterile *Y*'s and the technique of inducing them are being used in a cytogenetic analysis of the *Y* chromosome.

4. Yeast, Hymenoptera, and Phage Genetics

R. C. von Borstel[a]	
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Insect Cytology and Genetics (4.2–4.4) R. L. Amy[a,b] J. G. Carlson[b] Mary E. Gaulden[b] Roger H. Smith[a,c] Anna R. Whiting[a,b] P. W. Whiting[b] Margaret H. Lane[a] Martha S. Jones[a] Joan W. Reel[a] Dale E. Graham[d] Kathleen J. La Brot[e] Kathryn Murray[e] Julie T. Tindall[a] Judith W. Steen[a]	***Participation in Cooperative Programs:*** **AEC-NASA Space Biology (26.56, 26.57)**
[a]Dual assignments [b]Consultant [c]Research associate	[d]National Science Foundation Traineeship Loanee [e]Student trainee

4.1 THE ENHANCED SPONTANEOUS MUTATION RATE OF AN X-RADIATION-SENSITIVE YEAST

R. C. von Borstel
Kathleen J. La Brot
Dale E. Graham
Michael A. Resnick[1]

In an attempt to understand the nature of an x-radiation-sensitive locus in yeast, we elected to measure the spontaneous reversion rates of supersuppressible mutants of structural genes and forward mutations of their suppressors. By this means, reversion specificities could be sought.

The common genotypes of the strains used in this study are ar_{4-17}, hi_{5-2}, ad_{2-1}, and le_{1-12}. These four are suppressible by a number of different supersuppressor genes, and the le_{1-12} mutant is complementing. Suppressors that revert to prototrophy all four of the supersuppressible mutants are called class I supersuppressors; those not reverting the ad_{2-1} gene, but reverting the others, are called class II supersuppressors.

Strain KC 376, the x-radiation-sensitive strain, was derived in one step from the X1687-101B strain as a mutant induced by nitrous acid.

The exposure-survival curves of the x-radiation-sensitive strain and the control strain are shown in Fig. 4.1.1. The most noticeable difference between the two curves is the abrupt change of slope,

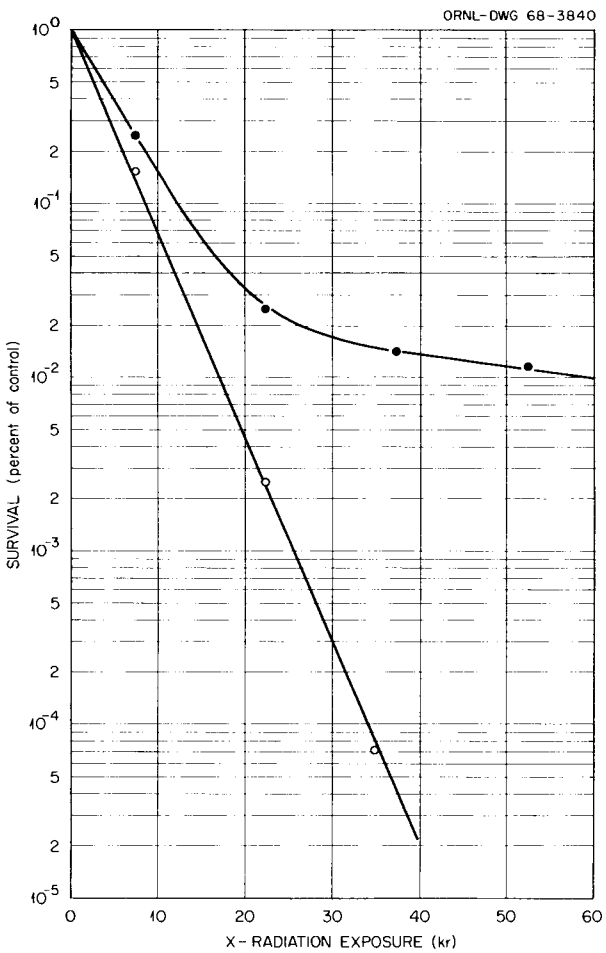

Fig. 4.1.1. Survival of Yeast at Different Exposures of X Radiation. ● = strain X1687-101B, a genetically marked strain with wild-type properties of response to radiation; O = strain KC 376, an x-radiation-sensitive strain.

indicating the presence of a radiation-resistant cell population in the control strain. This resistance to x radiation which is observed in yeast cells undergoing division is lacking in the x-radiation-sensitive strain.

When the spontaneous mutation rates for reversion from two different auxotrophic requirements were determined, it was found that the class I supersuppressors of the x-radiation-sensitive strain mutate at a rate approximately 40 times that of the control, while the class II supersuppressors and the structural gene itself mutate at approximately twice that of the control (Table 4.1.1).

Since the great enhancement of mutation rate is restricted to the class I supersuppressors, it is of interest to discuss these particular genes. The class I supersuppressors are genes believed to encode tRNA. This belief is based upon (1) genetic grounds, where it has been argued that at least some of the supersuppressors encode tRNA,[2] and (2) biochemical grounds, where it can be stated that $S\alpha$, a supersuppressor of the class I series, encodes either a tRNA or a tRNA modifying enzyme. The tRNA from the strain containing $S\alpha$ inserts an amino acid into a protein chain in a position encoded by a nonsense (UAG) triplet (Wilhelm and von Borstel, unpublished data).

The enhancement of mutation rate during meiosis is associated with recombinational events during meiosis; it is consistent with the argument that these are base additions or deletions arising from unequal crossing-over. Further, mutations presumed to be base substitutions do not have a greatly enhanced mutation rate during meiosis; supersuppressible mutants belong to this class.

Thus the data are consistent with the notion that the enhanced spontaneous mutation rate of the class I supersuppressors in the x-radiation-sensitive strain arises largely from an increase in the frequency of occurrence of addition-deletion mutations or a decrease in the frequency of repair of potential addition-deletion mutations.

References

[1]Donner Laboratory, University of California, Berkeley, Calif.

[2]G. E. Magni, R. C. von Borstel, and C. M. Steinberg, *J. Mol. Biol.* **16**, 568–70 (1966).

4.2 DOMINANT LETHAL MUTATIONS AND TEMPERATURE-SENSITIVE RECESSIVE LETHAL MUTATIONS INDUCED BY ETHYL METHANESULFONATE IN *HABROBRACON* SPERM

Roger H. Smith

Ethyl methanesulfonate (EMS), a highly mutagenic compound that induces missense mutations in T4 phages,[1] was fed to *Habrobracon* males to induce dominant and recessive lethal mutations in sperm. Recessive mutations can be detected for the entire genome by breeding females unmated; these produce haploid male offspring.

Table 4.1.1. Spontaneous Mutation Rates for Genes Involving Single Auxotrophic Requirements and for Supersuppressibility

Strain	Sensitivity	Growth-Limiting Medium	Prototrophs per Cell per Generation		
			Structural Gene	Class I Suppressors	Class II Suppressors
			$\times 10^{-8}$	$\times 10^{-8}$	$\times 10^{-8}$
X1687-101B	Control	Lysine (Expt. 1)	1.6	1.1	11.6
		Lysine (Expt. 2)	1.3	1.7	9.3
		Arginine	2.6	0.9	5.4
KC 376	X-radiation sensitive	Lysine (Expt. 1)	2.4	28.4	8.0
		Lysine (Expt. 2)	6.3	77.3	17.6
		Arginine	4.8	43.1	12.8

Males of the Lumberton (lum D) strain were fed a sucrose solution containing EMS dissolved in phosphate buffer at pH 7.0. Three concentrations were used, 0.1, 0.05, and 0.01 *M*. The males were placed in an incubator at 30°C for 24 hr, and observed matings were made to females of the Raleigh (R) strain. Both strains had been cleared of all spontaneous recessive lethal mutations in the previous generation.

The temperature-sensitive recessive lethals were found by placing about one-half of the eggs from each female at 35°C to develop to the adult stage. The other half were placed at the normal incubation temperature.

The males which fed on the 0.1 *M* solution were weak, and only four of them were observed to mate. The eggs from these crosses had high hatchability, and no female offspring were produced. This suggests that there was either inactivation of sperm or lack of an ejaculate from the weakened male.

Table 4.2.1 shows that concentrations of 0.05 and 0.01 *M* EMS induced dominant lethal mutations in sperm. The highest of these two concentrations induced essentially 100% dominant lethality.

Table 4.2.2 shows the pattern of recessive lethal mutations induced by EMS. Nearly twice as many lethals are expressed during the larval period as during embryogenesis. The incidence of lethals that are temperature sensitive is about the same for each developmental period. This incidence is similar to that found by Suzuki and his collaborators,[2] where about 10% of the sex-linked lethals induced by EMS in *Drosophila* sperm are temperature sensitive.

Table 4.2.1. Survival of Diploid Embryos After Treatment of *Habrobracon* Sperm by Feeding Ethyl Methanesulfonate in Sucrose

EMS Concentration	Total No. Eggs	Survival to Blastula	Survival to Larvae	Survival to Adult Female
0.05	413	0.095	0.003	0
0.01	1384	0.865	0.756	0.509
Buffer	577	0.983	0.952	0.662
Sugar only	400	0.988	0.941	0.612

References

[1]D. R. Krieg, *Genetics* **48**, 561–80 (1963).
[2]D. T. Suzuki *et al.*, *Proc. Natl. Acad. Sci. U.S.* **57**, 907–12 (1967).

4.3 ELECTROPHORETIC VARIANTS OF XANTHINE DEHYDROGENASE IN GEOGRAPHICAL RACES OF *HABROBRACON*

Roger H. Smith Kathryn Murray

Strains of *Habrobracon juglandis* from various parts of the United States were analyzed for possible variants of the enzyme xanthine dehydrogenase (XHD). This enzyme catalyzes the oxidation

Table 4.2.2. The Frequencies of Recessive Lethal Mutations Induced by 0.01 *M* Ethyl Methanesulfonate

Type	Embryonic	Larval	Pupal	Total
Standard	62/437 = 0.142	114/437 = 0.261	45/437 = 0.103	221/437 = 0.506
Temperature sensitive	7/62 = 0.11	11/114 = 0.096	5/45 = 0.11	23/221 = 0.104

of xanthine and hypoxanthine to uric acid. A difference in electrophoretic mobility for XDH between two species of *Habrobracon*, *H. juglandis* and *H. serinopae*, was reported earlier.[1]

The city (when known) and state where the different strains were caught are presented in Table 4.3.1 along with the allele designations for the XDH-determining locus. The alleles are named for their relative mobilities.

Reciprocal crosses were made between R and the other strains. The hybrid females of each cross produced a single wide band which corresponded on its fastest and slowest boundaries to the fastest and slowest boundary of the other parental band. Unsuccessful attempts were made to resolve the F_1 band into two or more bands by various changes in pH and molarity of the electrode buffer. Also, individuals of the different strains were homogenized together, and the electrophoretic mobility of the mixture determined; the results were identical to those obtained from the hybrids between the corresponding strains. The haploid male offspring (F_2) from these crosses produced bands like one or the other of the original grandparents, indicating simple Mendelian inheritance.

Table 4.3.1. The Location of the Strains of *Habrobracon juglandis* Tested and the Gene Designation for Xanthine Dehydrogenase

Strain Designation	Location	Allele Designation
R	Raleigh, N.C.	$xdh^{1.00}$
nat (4 lines)	Natchitoches, La.	$xdh^{1.00}$
nat No. 2	Natchitoches, La.	$xdh^{0.98}$
lum D	Lumberton, N.C.	$xdh^{0.97}$
Davis	Davis, Calif.	$xdh^{0.93}$
No. 33	Pennsylvania	$xdh^{0.91}$

Polymorphism for a particular gene (xdh) exists between widely separated populations and within at least one population (Natchitoches) of *Habrobracon juglandis*.

Reference

[1]R. H. Smith and L. R. Valcovic, *Biol. Div. Semi-ann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, p. 64.

4.4 CYTOCHEMICAL LOCALIZATION OF SITES OF RNA SYNTHESIS USING RNA POLYMERASE

R. C. von Borstel
Joan W. Reel
S. P. Modak[1]
F. J. Bollum[2]

Previous work with DNA polymerase[3] showed that an alcohol-fixed, acid-denatured tissue preparation catalyzed a net amount of DNA synthesis. Since DNA polymerase from calf thymus cannot use native DNA as a template, this method helps localization of single-stranded DNA in fixed cell preparations. It was conceived that a similar qualitative method of the localization of sites of RNA synthesis can be developed using RNA polymerase.

RNA polymerase from *Micrococcus lysodeikticus* catalyzes a net amount of RNA synthesis when native or denatured DNA is used as a template. The RNA that is synthesized with native DNA as a template is loosely bound, but when denatured DNA is used as a template, a complex of RNA and DNA is formed. Considering these features, we have developed a method for distinguishing the state of DNA molecules and for localization of sites of RNA synthesis in fixed cell preparations using RNA polymerase.

Adult chicken blood smears and a variety of other cell types have been used for this investigation. Cell preparations were made on cover glasses, fixed in cold 70% ethanol, and air-dried. Cover glasses with cell preparations were stored at 8 to 10°C. Glass slides with holes punched in the cen-

ter (6 mm in diameter) were attached to the cover glass in such a way that the cells were facing the hole. These now served as a well, with cells at the bottom. Half the cell preparations of each type were treated with 0.1 *N* HCl for 30 min at room temperature in order to denature DNA. Both undenatured and denatured cell preparations were individually treated with either RNA or DNA polymerase and labeled nucleoside triphosphates. Some preparations were treated with the reaction mixtures without the inclusion of either enzyme, and these served as controls for determination of any unspecific localization of radioactivity. Preparations were incubated with the reaction mixture for 60 min. Enzyme reaction was stopped by rinsing the slides in saline and then in 5% cold TCA containg 1 m*M* Pi. After one change, slides were left in cold TCA overnight and then thoroughly washed in running water. Some of the RNA-polymerase-treated preparations were then treated with pancreatic ribonuclease (5 mg/ml RNase in 0.2 *M* NaCl, buffered *M*/15 Pi at pH 7.0 for 10 min at room temperature). These were quickly rinsed in NaCl solution and passed through two 5-min changes of 5% cold TCA. All TCA-treated slides were thoroughly washed in running distilled water, dehydrated up to 95% ethanol, and air-dried. Coverslips were individually detached from the glass slides and reattached to fresh intact slides with Canada balsam leaving the cells facing upward. These were well dried and then coated with NTB-3 (Kodak) liquid emulsion. Autoradiograms of DNA- and RNA-polymerase experiments were developed after 14 and 35 days, respectively, in D-11 developer. Cells were counterstained with Mayer's hemalum, dehydrated, and mounted in Canada balsam. The scheme of the experiment is shown in Fig. 4.4.1.

The observation of autoradiographs revealed the following features:

1. Cells fixed in 70% ethanol only and then treated with ^{3}H-NTP's did not incorporate any radioactivity, and the background was very low or negligible.
2. Cells fixed in 70% ethanol and treated with ^{3}H-NTP's and RNA polymerase showed some radioactivity over the nuclei and a very high grain count over the area surrounding or away from the nuclei. Most of the radioactivity was removed by RNase treatment.

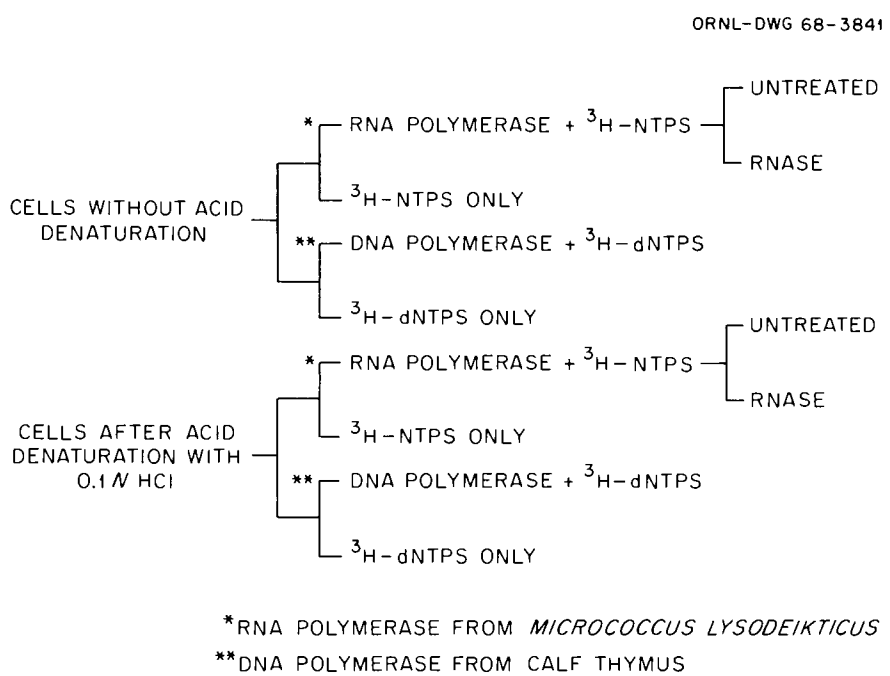

Fig. 4.4.1. Autoradiographic Analysis of Incorporation of Tritium-Labeled Nucleoside Triphosphates During Polymerization of DNA and RNA.

3. 70% ethanol-fixed and acid-denatured cells treated with nucleoside triphosphates only did not incorporate the isotope.

4. 70% ethanol-fixed and acid-denatured cells incubated with ^{3}H-NTP's and RNA polymerase showed a very high incorporation and background grain count. Most of the background grain count and a large proportion of grains over the nuclei were removed by RNase. However, some grains persisted over the nuclei even after RNase.

5. No incorporation of tritium was observed when cells were left undenatured and treated with DNA polymerase. However, on acid denaturation, significant incorporation of ^{3}H-dNTP's was observed over the nuclei.

The results clearly show that one can demonstrate incorporation of labeled RNA precursors using RNA polymerase in fixed cell preparations. On the basis of digestion with RNase of the incorporated radioactivity, one can predict the state of DNA molecules in fixed cell nuclei. From our results, there is no single-stranded DNA in chicken blood nuclei and a variety of other cell types, and most of the RNA catalyzed by RNA polymerase is loosely bound to the native DNA. On acid denaturation, however, a significant amount of newly synthesized RNA remains as an RNA-DNA complex and is RNase resistant.

The method can be a useful tool in determining the degree of denatured state of DNA in fixed cell populations. Given a large enough chromosome preparation, by combining RNA polymerase treatment with RNA hybridization procedures, one could conceivably demonstrate sites of synthesis for different RNA species. Some such experiments using *Drosophila* and *Chironomus* salivary gland chromosomes are now in progress.

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[1]Cell Growth and Differentiation section.

[2]Department of Biochemistry, University of Kentucky Medical Center, Lexington.

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4.5 INTERFERENCE IN CIRCULAR GENETIC MAPS

C. M. Steinberg Franklin W. Stahl[1]

The mapping function for a conventional genetic map free of interference was derived by Haldane,[2] who recognized that freedom from interference implies a Poisson distribution of crossovers among strands. This hypothesis leads to the following well-known relation between R, the recombinant frequency for a pair of markers, and l, the map distance between the markers:

$$R = \frac{1}{2}(1 - e^{-2l}).$$

For the hypothetical case of crossing-over in a two-strand stage, l is simply the mean number of crossovers between the markers, and R the sum of the odd terms of a Poisson distribution of mean l.

Clearly, this null hypothesis and mapping function are inappropriate for circular genetic maps. In the case of circular maps, only those strands are recovered which have engaged in an even number of crossovers; therefore, the "expected" distribution of crossovers cannot be Poisson. We have proposed[3] for circular maps three simple mapping functions which adhere closely to the spirit of the classical function. Each of the functions derives from a different simple assumption regarding the distribution of crossovers among (recovered) strands: (1) a Poisson distribution of pairs of crossovers, (2) a Poisson distribution of crossovers from which the strands receiving an odd number of crossovers are dropped, and (3) a Poisson distribution of crossovers with an additional crossover disposed at random on each of the strands which initially received an odd number.

Distribution 2 leads to generally useful definitions of "no interference" that are equally applicable to linear and circular maps.

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[1]Department of Biology and Institute of Molecular Biology, University of Oregon, Eugene.

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[3]C. M. Steinberg and F. W. Stahl, *J. Cell Physiol.* **70**, suppl. 1, 4–7 (1967).

5. Cellular Radiobiology and International Activities

Alexander Hollaender[a] Mary Jane Loop

[a]Dual assignments.

The first two and one-half months of 1967 were spent on a trip abroad visiting European laboratories and Thailand, Pakistan, and India in the Far East. Arrangements have been made with Pakistan for a Molecular Biology Course to be given by members of the Biology Division, as well as others, beginning in the Fall of 1968, to be financed entirely by the Pakistan Atomic Energy Commission. Also, arrangements were made for a possible symposium at the conclusion of this course in 1969.

A considerable number of laboratories in northern India were visited and arrangements made for a Symposium on Fundamental Radiobiology to be held at the Bhabha Research Centre in February 1969. Also, a number of contacts were made and preparations begun for cooperation with Indian scientists.

In July, at the invitation of the Brazilian Academy, three members of the Biology Division (Russell, Upton, and myself) attended a meeting on Peaceful Aspects of Atomic Energy held in Rio de Janeiro. The papers of this symposium are being published by the Brazilian Academy in a special volume. During this visit an inspection trip was made to Belo Horizonte to prepare for the 1968 symposium.

In October of 1967 the Ford Foundation contributed a substantial amount of funds for two years for the support of these Latin American symposia. The money is to be used to support Latin Americans to attend these symposia, a modest amount of money is for the travel of the organizers for future symposia, and also some contribution is made toward the editing and publication of these symposia. This grant is being administered by the Latin American group in the Office of the Foreign Secretary at the U.S. National Academy of Sciences.

In November and December of this year visits were made to Sao Paulo and two cities in the Nordeste area of Brazil, Recife and Fortaleza, an important section of Brazil which has been neglected for many years and is now coming to the forefront in industrial and educational activities.

The Seventh International Latin American Symposium on Photochemistry and Photobiology was held in Caracas in December. It was a very successful meeting with many Latin Americans attending. The proceedings of this symposium are being prepared for publication. The proceedings of the 1966 Mexico City symposium on Enzymatic Aspects of Metabolic Regulation have been published as National Cancer Institute Monograph 27, November 1967.

We cooperated with the Puerto Rico University Biology Department in organizing a course in Modern Biology. The course is now being given at Rio Piedras with Dr. Billen from the University of Florida in charge, and with one professor from the University of Texas at Houston, one from Pennsylvania State University, and two scientists from the Biology Division, Oak Ridge National Laboratory, each giving three weeks of lectures and laboratories.

Considerable effort was put into trying to convince pharmacologists, as well as other people, of the importance of chemical mutagenesis. It could

very well become a kind of focal point for further development of cooperation with different government agencies in building up new approaches to basic biology using the same type of approach which has been used in the radiation field. These projects are still under discussion.

Considerable effort was spent on developing the idea of setting up a long-term planning institute in biomedical and health sciences. A prospectus was prepared, several meetings were held, and the National Academy of Sciences is now considering possible sponsorship of this institute with the cooperation of a number of outstanding universities.

6. Radiation Microbiology and Microbial Genetics

H. I. Adler[a]

Radiation Microbiology and Microbial Genetics

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6.1 CONTROL OF CELL DIVISION IN *ESCHERICHIA COLI*

H. I. Adler Alice A. Hardigree

We have recently described a mutant strain of *E. coli* K-12 that, due to a defect in its cytokinesis mechanism, produces large numbers of small, enucleate "minicells."[1] The divisional process that yields a minicell is similar to normal cell division in at least two ways: (1) It involves a simultaneous invagination of wall and membrane when observed by electron microscope techniques and (2) when observed by phase microscopy, it can be concluded that the time required to complete a minicell-yielding division is similar and probably identical to that required for a division yielding normal cells. Minicell-yielding divisions differ from normal cell division in that (1) they occur very close to the poles of the cell and (2) nuclear material is not distributed to both sides of the developing cross wall. We are interested in determining the extent to which both kinds of cytokinesis are under the same genetic and biochemical control.

For this purpose, we have constructed a double mutant incorporating the minicell-producing feature (min^-) into an organism that has had its normal cell-division mechanism impaired (lon^-) and therefore forms long filaments after response to ionizing radiation. The double mutant forms minicells and normal cells when grown in the absence of radiation. After exposure to radiation, cells of the double mutant do not undergo either minicell- or normal-cell-yielding divisions. They do, however, continue to grow for many hours and form long multinucleate filaments. These results

indicate that both kinds of cytokinesis share at least some common pathway, and the lesion caused by irradiation interferes equally with both processes.

Reference

[1]H. I. Adler *et al.*, *Proc. Natl. Acad. Sci. U.S.* **57**, 321–26 (1967).

6.2 THE ISOLATION OF DNA FROM MINICELLS AFTER MATING WITH F^+ CELLS

Amikam Cohen W. D. Fisher[1]
H. I. Adler

Since *Escherichia coli* minicells do not contain any DNA,[2] one can use them as recipients in bacterial conjugation in order to isolate specific segments of the bacterial genome.

Minicells were separated from cultures of *E. coli* P-678-54 by differential centrifugation and then mated with F^+ cells. The DNA which was transferred during mating was then extracted from the purified minicells, and some of its properties were studied. In such matings, only the F factor is transferred at high frequency; therefore the DNA isolated must represent a sample of this episome.

This DNA could be separated by neutral cesium chloride density gradient centrifugation into two components, one which consists of one-fourth to one-third of the total material, bands at a density of 1.71, and coincides with native *E. coli* DNA marker, and another which contains most of the material, bands at a density of 1.72, and coincides with denatured *E. coli* DNA marker; both fractions band as one peak in alkaline cesium chloride and coincide with *E. coli* DNA.

The material isolated at a density of 1.72 separates from native and coincides with denatured *E. coli* DNA in a cochromatography on a hydroxyapatite column and is partially digestible with *E. coli* exonuclease I. After shearing by sonication, 90% of this material is digestible with exonuclease I. These results indicate that it may have some secondary structure, but most of it is single-stranded DNA.

When minicells are isolated after mating and incubated for 30 min or less in a growth medium, most of the material associated with the single-stranded DNA fraction disappears, and the amount associated with the double-strand fraction increases; if labeled thymidine is added during this period, it is incorporated only into the double-stranded material.

These results are consistent with the hypothesis that in bacterial conjugation DNA is transferred as a single strand and then replicates in the recipient.

Presently we are studying some of the physical properties of the transferred material, and we are trying to determine whether the single-stranded fraction consists of a unique strand or a mixture of two complementary strands.

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[1]General Physiology section.

[2]H. I. Adler *et al.*, *Proc. Natl. Acad. Sci. U.S.* **57**, 321–26 (1967).

6.3 GIANT CELLS OF *ESCHERICHIA COLI*

H. I. Adler C. E. Terry
Alice A. Hardigree

One of the striking expressions of mutation at the "lon" locus in *Escherichia coli* is the enhanced sensitivity of cytokinesis to radiation and other agents. Cells of strains bearing this mutation grow after irradiation but fail to divide. The mutation has previously been studied only in rod-shaped organisms, in which growth without division leads to the formation of extremely long filaments. We recently isolated an *E. coli* mutant that consists of amorphous cells. This gave us the opportunity to study the effect of the "lon" gene in a new genetic background. The mutant "lon" allele was introduced into the amorphous strain by conjugation with lon^- donors. We have observed that cells of the double mutant, after exposure to radiation, grow without division into giant amorphous forms that are analogous to the filaments formed from rod-shaped lon mutants. The giant cells may attain volumes 500 to 1000 times that of a normal *E. coli* cell. They, like filaments, grow at a normal rate for several hours after irradiation but then stop growing and do not divide. During the period of active growth, many spherical structures appear, coalesce, and frequently disappear. Other internal bodies have been noted but not identified. Preliminary chemical data

indicate that the giant cells are multinucleate. We anticipate that these cells will be useful in the probing of the structural organization of bacteria.

6.4 THE INFLUENCE OF AN EPISOME ON THE SENSITIVITY OF *ESCHERICHIA COLI* K-12 TO IONIZING RADIATION

D. E. Axelrod H. I. Adler

Previous work from several laboratories has demonstrated that the sensitivity of bacteria to ionizing and ultraviolet radiations is under the control of many chromosomal genes. Several of these have been mapped.[1] In the present study we have demonstrated that sensitivity to ionizing radiation may also be influenced by an episome, the F factor of *E. coli* K-12.

Sets of isogenic strains consisting of high-frequency donors (Hfr) in which F is integrated in the chromosome, low-frequency donors (F^+) in which F is extrachromosomal, and recipient strains (F^-) in which the F factor is absent from the cell have been constructed. The order of resistance for several such isogenic series is $Hfr > F^- \geq F^+$. The resistance of an Hfr strain compared with its isogenic F^+ strain is independent of the map location of the integrated episome and independent of the direction of chromosome transfer. Transfer of the F factor by Hfr strains to F^- strains confers radiation resistance upon those recombinant progeny in which the episome is integrated into the chromosome.

Reference

[1]H. I. Adler, in *Advances in Radiation Biology*, vol. 2 (ed. by L. G. Augenstein, R. Mason, and M. R. Zelle), Academic, New York, 1966.

6.5 TIME-LAPSE CINEMATOGRAPHY OF NUCLEAR DIVISION IN BACTERIA

H. I. Adler Alice A. Hardigree

The relationships between DNA replication, nuclear division, and cell division in bacteria are now being explored in many laboratories. For the most part, indirect biochemical and genetic techniques are being employed. We have decided to find out how much we can learn by direct phase-microscopic observation on living cells. It is clear that such an approach cannot yield molecular information, but it should certainly be capable of setting limits within which the results of biochemical and genetic experiments must fall.

In order to see the nuclear region of bacteria with phase-microscopic techniques, it is necessary to adjust the refractive index of the medium in which the cells are suspended so that it nearly matches that of the cell cytoplasm.[1] Under these conditions, nuclei appear as bright objects in a dark background when dark phase contrast equipment is used. We have achieved the necessary adjustment of refractive index by incorporating 20 to 26% polyvinylpyrrolidone (PVP) into nutrient agar. *Escherichia coli* cells grow at a nearly normal rate in this medium. Thin layers of PVP nutrient agar are poured onto microscope slides. After the agar has set, cells from a PVP–nutrient-broth culture are spread on the surface. A cover slip is placed over the agar and the specimen observed at 37°C with a Zeiss 100X oil-immersion dark-contrast objective. Using this technique, we have been able to observe nuclear and cell division in several strains of *E. coli*. Most strains appear to be binucleate during the logarithmic phase of growth, but differences in nuclear morphology have been noted. We have also been able to follow the replication of nuclei in the absence of cell division by observing the long filaments formed by certain mutants of *Escherichia coli* after exposure to ionizing radiation. Currently, we are attempting to solve the technical problems of adapting this technique to our time-lapse cinematographic equipment. We have produced films that record nuclear division, but to date the quality of the photographic image is far below that observed directly. It should be possible to correct this problem by the appropriate choice of films and exposure conditions.

Reference

[1]D. J. Mason and D. M. Powelson, *J. Bacteriol.* **71**, 474–79 (1956).

6.6 CONJUGAL TRANSFER OF F′ FACTORS OF *ESCHERICHIA COLI* INTO *PROTEUS MIRABILIS*

G. J. Stine Annie S. Angel
Roy Curtiss III

The purpose of this study is to develop techniques for isolation of specific gene fragments from *Escherichia coli* K-12. The initial approach was to transfer the F′ elements of *E. coli* K-12 strain χ517 F′ *lac*$^+$ *proC*$^+$ *purE*$^+$ into the appropriate mutant derivative of *Proteus mirabilis* by conjugation. The necessary *P. mirabilis* mutant strains have been isolated by ultraviolet and nitrosoguanidine mutant induction procedures. A *P. mirabilis* strain (Pm 4-38) deficient in the ability to utilize lactose (lac$^-$) and unable to synthesize proline (pro$^-$) and adenine (purE$^-$) was used as the recipient in the mating with χ517. The isolation of recombinants from the χ517 × Pm 4-38 mating yielded a strain of *P. mirabilis* (Pm 4-38-5) which carries the F′ element of χ517. *E. coli* DNA has a 50% G-C content and *P. mirabilis* DNA a 38% G-C content, and genetic exchange does not occur between the *E. coli* F′ factor and the *P. mirabilis* chromosome. Thus it is possible to separate the *E. coli* DNA from the *P. mirabilis* DNA either by density gradient centrifugation or by differential melting followed by column chromatography. The separation and purification of the gene fragment from χ517 (F′ *lac*$^+$ *proC*$^+$ *purE*$^+$) now contained in *P. mirabilis* Pm 4-38-5 will begin immediately. The isolated *E. coli* DNA, which is enriched for the specific segment of the genome, will be tested for its ability to hybridize with *E. coli* transfer RNA. Results of these physical hybridization mapping experiments will determine the feasibility of other experiments such as the in vitro synthesis of biologically active transfer RNA from a DNA template and determination of whether all transfer RNA's which code for the same amino acid are necessary.

6.7 BACTERIAL CONJUGATION WITH Hfr DONORS OF *ESCHERICHIA COLI* K-12

Roy Curtiss III

Most of our efforts during the year have been directed toward a further understanding of the mechanisms operative during conjugal transfer of genetic material from Hfr and F$^+$ (see following report) donors to F$^-$ recipients of *Escherichia coli* K-12. The experiments completed have for the most part strengthened conclusions established by earlier work, but in several cases they have resulted in a modification and/or rejection of prior conclusions.

Specific Pair Formation (with D. R. Stallions, D. P. Allison,[1] and L. G. Caro[1]). – Formation of specific pairs between donor and recipient cells of *E. coli* K-12 is the first step during bacterial conjugation. Experiments completed during the year have corroborated the conclusion that F pili[2] are necessary for specific pair formation[3] and that specific pairs are formed in the absence of energy metabolism in both parents.[4]

Conjugation Tube Formation (with D. R. Stallions, L. G. Caro,[1] D. P. Allison,[1] and Amikam Cohen). – The conversion of a specific pair to an effective pair requires the establishment of a conjugal bridge between the donor and the recipient cell. An effective pair is thus defined as one in which chromosome transfer can or is taking place. Our previous work had led us to agree with Brinton[5] that F pili act as conjugation tubes, in addition to their role in specific pair formation.[6] The following observations lead us to now believe that F pili do *not* act as conjugation tubes. (1) Hfr donors having numerous, long F pili transfer the lead marker on their chromosomes sooner after commencement of mating than do Hfr donors having fewer, short F pili. If the chromosome had to pass through the F pilus, then initial marker transfer should take longer for donor cells with longer F pili. (2) Preliminary observations suggest that the amount of labeled DNA transferred to minicells is proportional to the number of minicells closely paired with donor cells as scored by electron microscopy. (3) Observations by light microscopy of morphologically distinct donor-recipient pairs impinged on slides covered with an agar medium selective for recombinants have shown that genetic transfer takes place when the donor and recipient are in close contact with each other. No instances were seen which would indicate genetic transfer between cells not in close contact. (4) Mutants of donor strains which are resistant to donor-specific bacteriophages have been isolated and characterized. Most of these lack F pili and are incapable of pair formation with F$^-$ cells. However, the recipient ability of some of these mutants

in matings with donor strains is equal to that of F^- strains, while others have the low recipient ability characteristic of wild-type donor strains. Mutants in the latter group also remain resistant to the quasi-recipient-specific bacteriophage ϕII. These observations suggest that donor strains possess a cell surface component, in addition to F pili, which prevents donor cells from mating with each other. (5) Mutants of Hfr donors which are resistant to phages T3, T4, T7, and λ produce a mucoid capsular material and are incapable of producing recombinants when mated with F^- strains.[7] Some of these mutants have F pili and others do not. Presumably the capsular material covers the donor cell surface component in the former class, which leads us to suggest that the donor cell surface component is necessary to convert a specific pair into an effective pair and that the presence of F pili is not sufficient to accomplish this.

The above observations are consistent with the belief that (1) F pili do not function as conjugation tubes and (2) a donor cell surface component prevents close cell-to-cell union between donor strains and is necessary for close cell-to-cell union between donor and recipient strains. Based on the properties of the mutant donor strains isolated, we believe that the presence of this donor cell surface component is under the control of genes present in the fertility factor. The relationship between this donor cell surface component and the establishment of a conjugal bridge is presently under investigation.

Requirement for Genetic Homology Between the Lead Region of the Hfr Chromosome and the Comparable Segment of the F^- Chromosome (with D. R. Stallions and Dale Graham[8]). – We[9,10] previously concluded that early initiation and maximum frequencies of chromosome transfer required genetic homology between the lead region of the Hfr chromosome and the comparable segment of the F^- chromosome. This year we have strengthened this conclusion by extending our earlier observations to include studies with several different sublines of *E. coli* K-12. We have also made use of a greater variety of deletion mutations in these studies.

The principal scientific objection to the conclusions drawn from these experiments has been that we have used recombinant production as a measure of chromosome transfer. Since recombinant production requires integration of the transferred donor chromosome it is conceivable that the deletions in the F^- chromosome interfered with this latter process. The Hfr strains heretofore available have precluded the possibility of using other means to measure chromosome transfer. Thus a major accomplishment this year has been the construction of donor and recipient strains which contain the inversion of the *lac* operon isolated and characterized by Berg and Curtis.[11] The presence of this inversion allows chromosome transfer in the opposite direction from that of donor strains used in our earlier studies, and we can now directly measure chromosome transfer, independently of recombinant formation, by using zygotic induction of prophage λ. The results from the experiments using these newly developed strains should provide convincing evidence for or against our postulate that early initiation and maximum frequencies of chromosome transfer require genetic homology between the lead region of the Hfr chromosome and the comparable portion of the F^- chromosome.

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[4]R. Curtiss III and D. R. Stallions, *J. Bacteriol.* **94**, 490–92 (1967).

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[6]Roy Curtiss III, J. A. Mays, and D. R. Stallions, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, p. 101.

[7]R. Curtiss III, *J. Bacteriol.* **89**, 28–40 (1965).

[8]Yeast, Hymenoptera, and Phage Genetics section.

[9]R. Curtiss III and L. J. Charamella, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1966*, ORNL-3922, p. 96.

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[11]C. M. Berg and Roy Curtiss III, *Genetics* **56**, 503–25 (1967).

6.8 BACTERIAL CONJUGATION WITH F^+ DONORS OF *ESCHERICHIA COLI* K-12

Roy Curtiss III D. R. Stallions

An overall review of our progress in understanding the mechanism of chromosome mobilization and transfer by F^+ donors of *Escherichia coli* K-12 was given in an earlier report.[1] A discussion of recent progress on the mechanism of F transfer is given in an accompanying report by Cohen *et al.*[2] Our own efforts this year have been directed toward answering three questions. First, we desired to understand the reason for the inability of type II F^+ donors to give rise to stable Hfr donors. Second, we wanted to directly measure the frequency of stable Hfr donors in populations of type I F^+ strains. Third, we wanted to determine what proportion of the recombinants in $F^+ \times F^+$ matings is due to stable Hfr donors present in the F^+ population.

Differences Between Types I and II F^+ Donors. – Type I F^+ donors are capable of giving rise to stable Hfr donors, whereas type II F^+ donors are not. Both F^+ donor types give equal recombinant frequencies when mated with the same F^- strains. The difference between these two F^+ donor types is not due to the presence or absence of λ, to differences in F, to environmental factors, or to any known genetic determinant.[3] During the year we have completed experiments which demonstrated that the difference in stable F integration ability was not due to differences in ability to support genetic recombination; F^- derivatives of types I and II F^+ donors are equally capable of recombinant formation when mated with F^+ and Hfr donors. To rule out the possibility that the presence of F in type II F^+ donors interfered with the normal recombination mechanism, we analyzed linked and unlinked marker transduction frequencies in a type II F^+ strain and in its Acridine Orange cured F^- derivative. There were no differences in P1*kc* mediated transduction frequencies observed. Since F integration into the chromosome requires a reciprocal recombination event, we next determined whether type II F^+ strains were capable of supporting reciprocal recombination. To do this we introduced F′ *lac*$^+$ episomes into *lac*$^-$ F^- derivatives of types I and II strains and compared the frequency of F′ *lac*$^+$ transfer with the frequency of chromosome transfer in matings with appropriately marked F^- strains. Since all strains behaved in the same way and chromosome transfer by partially diploid F′ donors requires a reciprocal recombination event between the F′ factor and the chromosome, we conclude that type II strains are not defective in supporting reciprocal recombination events.

During our studies of type I and type II F^+ donors, we noted that removal of F by growth in the presence of Acridine Orange was very efficient in type I F^+ strains and very inefficient in type II F^+ strains. A possible explanation for this behavior will be discussed below.

To determine whether F integration occurs but is lethal in type II F^+ strains, we introduced an F′ *lac*$^+$ episome, which contains a temperature-sensitive (*ts*) mutation affecting the autonomous replication of the episome, into *lac*$^-$ F^- derivatives of type I and II strains. At 37°C the F′ *lac*$^+$ episome replicates normally, while at 42°C it is unable to replicate unless the F′ *lac*$^+$ episome has been integrated into the chromosome by a reciprocal recombination event to produce an Hfr donor containing a tandem duplication of the *lac* genes. Following growth at 42°C *lac*$^+$ Hfr donors having a tandem duplication of the *lac* genes were obtained with type I strains but not with type II strains. Since F′ *lac*$^+$ and F′ *lac*$^+$ *ts* episomes do undergo reciprocal recombination with the chromosome of type II strains, as was determined by measuring the ability of such donors to transfer chromosomal markers (see above), we conclude that integration of the F′ *lac*$^+$ episome into the chromosome occurs but is lethal. Presumably the same conclusion would be true for F integration. To explain why F′ *lac*$^+$ and F integration would be lethal in type II strains, we postulate that the F and F′ episomes are linear, rather than circular, in type II strains. A reciprocal recombination event between a linear episome and a circular chromosome will result in a linear Hfr chromosome. If vegetative chromosome replication proceeds in a fixed direction from a fixed origin, then part of a linear chromosome would not be replicated, and thus a cell with such a linear Hfr chromosome could not produce any viable descendants. The difference in Acridine Orange curability of the F episome between types I and II F^+ strains could also be related to the proposed linearity of F and F′ episomes in type II strains.

The Frequency of Stable Hfr Donors in F^+ Populations and the Probability of F Integration into the Chromosome of *E. coli* K-12. – Integration

of the fertility factor F into the chromosome of *Escherichia coli* K-12 results in the formation of an Hfr donor. We have determined the probability of stable F integration per bacterium per generation and per chromosome per generation and the frequency of stable Hfr donors in populations of a prototrophic type I F^+ strain by replica plating more than 10^5 F^+ colonies to selective media spread with an F^- culture. (Stable F integrations were operationally defined as those which give rise to isolable clones of stable Hfr donors.) This method even allowed detection and isolation of Hfr donors transferring the selected marker on the distal 20% of their chromosomes and present at frequencies from 1 to 100% in F^+ colonies. Thirty-four Hfr strains were obtained, representing 12 unique marker transfer gradients. The frequency of pure clones of stable Hfr donors was 1.6×10^{-4}, and the probabilities of stable F integration were 3×10^{-6} per bacterium per generation and 8×10^{-7} per chromosome per generation.

The Proportion of Recombinants in $F^+ \times F^-$ Matings Due to the Presence of Stable Hfr Donors in the F^+ Population. – As reported in the above section, the frequency of stable Hfr donor cells in F^+ populations is between 1 and 2×10^{-4}. In order to determine the proportion of recombinants in $F^+ \times F^-$ matings which are due to these Hfr cells, matings were performed between an equal mixture of all 12 Hfr types isolated and a multiply marked F^- strain. The mean recombination frequency for any given chromosomal marker was found to be 1 to 2×10^{-2} per Hfr cell. In parallel matings using the F^+ strain from which all the Hfr donors had been isolated, the mean recombination frequency for any given marker was 1 to 2×10^{-5} per F^+ cell. By making use of all these frequencies it can be calculated that only about 10% of the recombinants formed in $F^+ \times F^-$ matings are due to stable Hfr donors in the F^+ population.

Although we believe[1] that 90% of the recombinants formed in $F^+ \times F^-$ matings are due to a mechanism which does not require F integration into the chromosome, we are presently conducting experiments to rigorously test this belief. In addition, we hope that the results obtained from these experiments will provide more information about the mechanism of chromosome mobilization and transfer by F^+ donors of *E. coli* K-12.

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7. Human Cytogenetics

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7.1 STATISTICAL ANALYSIS OF THE HUMAN KARYOTYPE

M. A Bender M. A. Kastenbaum[1]

In spite of general interest in the human karyotype, and in the problem of the identification of particular chromosomes, there has as yet been no satisfactory statistical description of the shapes and sizes of the chromosome set in the normal human population, nor any estimates of the importance of various possible sources of variation. A numerical description of what, precisely, constitutes a normal human chromosome set is obviously desirable, particularly in view of current efforts to program digital computers to analyze chromosome spreads. We have therefore made a statistical analysis of a large series of measurements of the chromosomes from cells of a series of 100 normal subjects.

Leukocyte cultures were made from blood samples from 50 male and 50 female normal volunteers. Metaphases suitable for karyotype analysis were selected and photographed. Each chromosome was identified with a code number on the enlargements. The length of each chromatid of each arm of each chromosome was then measured by means of a printing measuring device constructed for us by J. B. Davidson of the Instrumentation and Controls Division.[2] Independently, an experienced cytogeneticist prepared a karyotype of the cell by the conventional "cut and paste" method, and the chromosomes were each identified by code number. The assignments thus made were considered to be absolutely correct in the subsequent analyses.

Initially, a sample of ten cells was utilized from each of the donors. Preliminary statistical analysis of the as yet incomplete set of samples showed, however, that a smaller sample of cells from each individual would supply just as much information (vide infra), so the sample size was accordingly decreased in our later samplings. Altogether, measurements were accumulated from a total of 723 cells. Also, some of the cells were remeasured a number of times, both by the same and by different persons, to determine the magnitude of measurement errors. Finally, samples of cells from each of a series of separate cultures from a single donor were measured for the purpose of evaluating the importance of culture-to-culture variation.

A computer program (COVAL) was developed to normalize the measurements (as fractions of the total autosome length in the particular cell), to compute bivariate vectors of means, and to estimate simultaneous two-dimensional confidence regions for each chromosome pair (and also each group) in the human complement. Further statistical analyses of pooled data were carried out by standard covariance techniques.

It was found that while there were significant differences between the results of different individuals making measurements, the reproducibility for any single measurer was very good. Both the males and the females in the sample differed significantly in their mean chromosome measurements, although the variability among cells within each individual was not significantly different from the variability between pair mates within each cell. The differences between individuals appear actually to reflect a significant difference from culture to culture rather than real individual differences, since the differences between separate cultures from the same individual were not only highly significant, but the magnitudes of the vari-

Table 7.1.1. Pooled Means and Covariances for Male and Female Autosomes and X Chromosomes (1446 of Each Autosome; 1084 X Chromosomes)

Chromosome	Means			Covariance Matrix		
	N	Long	Short	Long	Short	Cross Product
1	1446	2.6173	2.2515	0.3221	0.1906	0.2134
2		2.8751	1.6984	0.2896	0.0773	0.1186
3		2.0333	1.6369	0.0885	0.0472	0.0327
4		2.6215	0.8689	0.2104	0.0302	−0.0455
5		2.4508	0.8087	0.1562	0.0219	−0.0304
6		2.0110	1.0902	0.0824	0.0275	−0.0062
7		1.7819	0.9811	0.0535	0.0238	−0.0111
8		1.6921	0.7940	0.0659	0.0292	−0.0270
9		1.6945	0.7366	0.0528	0.0341	−0.0168
10		1.6906	0.6456	0.0358	0.0304	−0.0213
11		1.4370	0.8039	0.0183	0.0273	0.0007
12		1.6706	0.5667	0.0399	0.0401	−0.0431
13		1.6529	0.2582	0.0314	0.0282	−0.0107
14		1.4201	0.2562	0.0292	0.0256	−0.0064
15		1.5113	0.2480	0.0263	0.0208	−0.0042
16		0.8957	0.5189	0.0510	0.0424	0.0308
17		0.9695	0.3693	0.0298	0.0397	0.0236
18		0.9055	0.2884	0.0427	0.0288	0.0218
19		0.5680	0.3905	0.0611	0.0510	0.0498
20		0.5269	0.3625	0.0621	0.0510	0.0508
21		0.5207	0.2081	0.0795	0.0212	0.0306
22		0.4728	0.1982	0.0693	0.0215	0.0314
X	1084	1.6989	0.9107	0.0663	0.0439	0.0069

ances among culture means were comparable with those among individual means.

The pooled means and covariances for the autosomes and the X chromosomes in the sample (both males and females) are shown in Table 7.1.1. In testing for differences between the male and the female means, significant differences were found only for the X ($p = 0.001$) and for chromosome 8 ($p = 0.01$). This result is not unexpected, since the X chromosome is most easily confused with the No. 8 chromosome pair, and since the method of identifying the X is necessarily different in male cells (where the X is "odd man out") from that in female cells. It is possible that the difference for the male and the female X also reflects a real difference in arm lengths in "active" and "inactive" X chromosomes.

Figure 7.1.1 shows a plot of the means of long and short arm measurements together with the 95% confidence regions for each chromosome. The confidence regions are small enough, because of the large sample size, so that only those for the F (19 and 20) and the G (21 and 22) group chromosomes come even close to overlapping. This

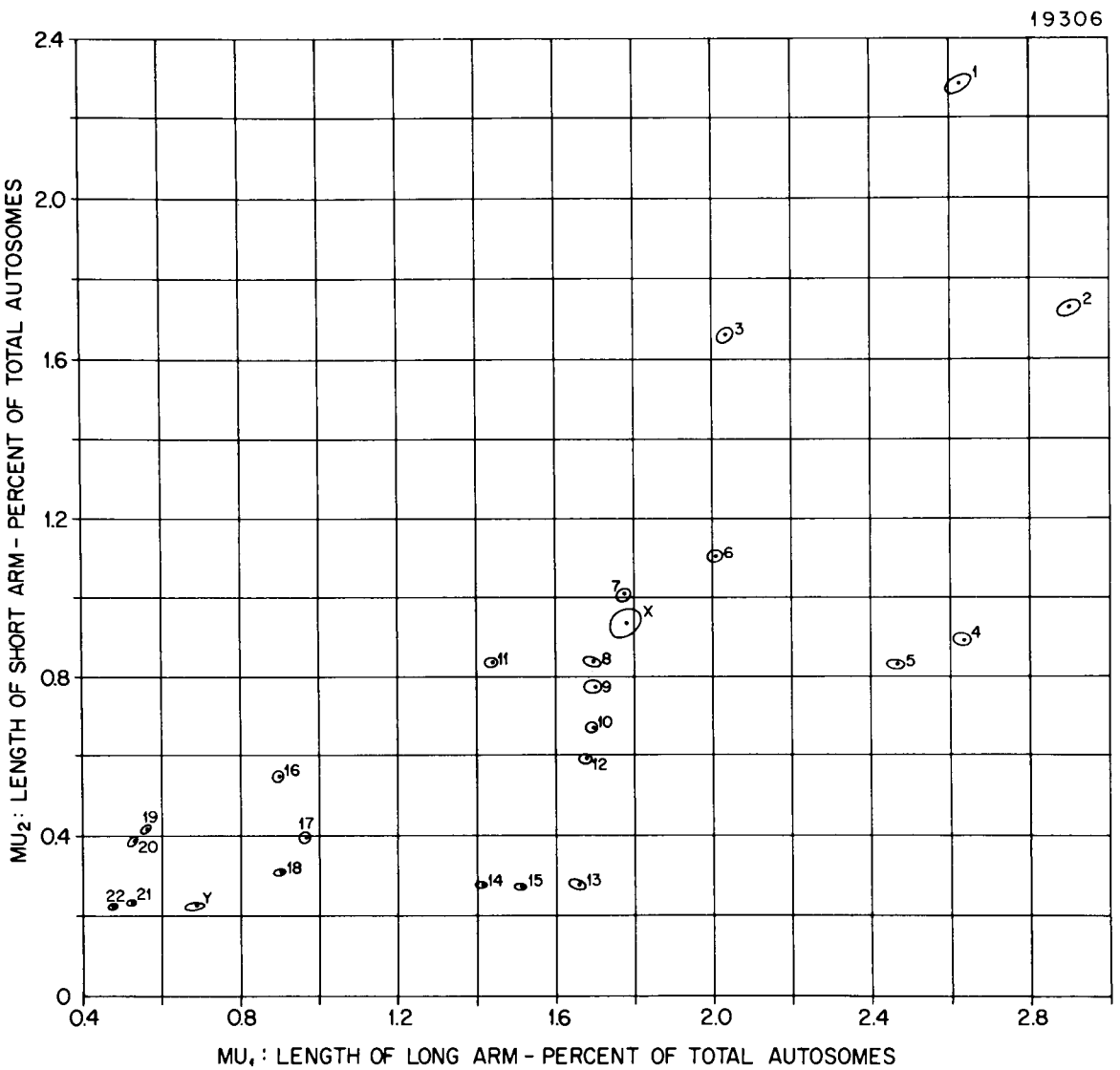

Fig. 7.1.1. Plot of Mean Chromosome Arm Lengths and Their 95% Confidence Regions.

should not, however, be taken as an indication of the "separability" of the various pairs from each other. The 95% tolerance regions, that is, the regions within which 95% of the individual pairs of measurements would fall, are considerably larger, and many of them do, in fact, overlap.

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7.2 PHOTOREACTIVATION AMONG THE CLASSES OF VERTEBRATES

James D. Regan John S. Cook[1]
W. H. Lee

Organ- and tissue-specific functions are lost from vertebrate cells after prolonged in vitro cultivation. Thus, a rigorous test of whether a given cellular property is tissue specific or is a fundamental cellular property common to all cells of the organism is the study of that property as it may exist in an established vertebrate cell line.

We have examined vertebrate cell lines for biological photoreactivation and for the presence of photoreactivating enzyme.

Our studies have thus far demonstrated that: (1) Teleost cells in vitro exhibit (*a*) photoreactivation of growth and DNA synthesis and (*b*) photoreactivating enzyme activity that can: (i) photoreactive *Hemophilus influenzae* transforming DNA, (ii) monomerize pyrimidine dimers in uv-irradiated *E. coli* DNA, and (iii) monomerize dimers in the DNA of the intact teleost cells.[2] (2) Amphibian cells in vitro exhibit (*a*) photoreactivation of growth and DNA synthesis and (*b*) photoreactivating enzyme in the *H. influenzae* transformation system. (3) Reptilian cells in vitro exhibit photoreactivation of cell growth. Further studies of reptilian cells are in progress.

Chick embryo fibroblasts in vitro possess photoreactivating enzyme[3] and can photoreactivate uv-irradiated pseudorabies virus,[4] but adult chick tissues exhibit little or no photoreactivating enzyme activity.[3] Thus it is conceivable that photoreactivation is a property recapitulated by birds during embryonic development and absent in adult birds. Studies of biological photoreactivation in avian cells are in progress using our experimental systems.

Mammalian cells in vitro apparently do not exhibit photoreactivation,[5] and neither fetal nor adult tissues of mammals show photoreactivating enzyme activity.[3]

From our studies and those cited, the distribution of this repair mechanism among the vertebrates presents several basic questions: (1) Why should a DNA repair mechanism, apparently highly fundamental on the cellular level to three classes of lower vertebrates, be absent in the highest class? (2) Does the presence of photoreactivation in bird embryonic cells but not in adult birds represent recapitulation? (3) If so, what is its evolutionary significance? (4) If the repair system is of fundamental necessity to fish, amphibia, reptiles, and embryonic birds, what system replaces it in adult birds and mammals?

Our continuing studies will be directed toward the elucidation of these questions.

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7.3 THE EXCISION OF PYRIMIDINE DIMERS FROM THE DNA OF ULTRAVIOLET-IRRADIATED HUMAN CELLS

James D. Regan W. L. Carrier[1]
J. E. Trosko[2]

In a previous report[3] we demonstrated that human cell lines, unlike Chinese hamster[4] and murine "L" cells[5] in vitro, lose about 50% of their ultraviolet (uv)-induced pyrimidine dimers during the first 24 hr after irradiation.

We have continued these studies and have demonstrated the following:

1. Dimer excision occurs in all the established human cell lines examined at all doses used (Fig. 7.3.1).

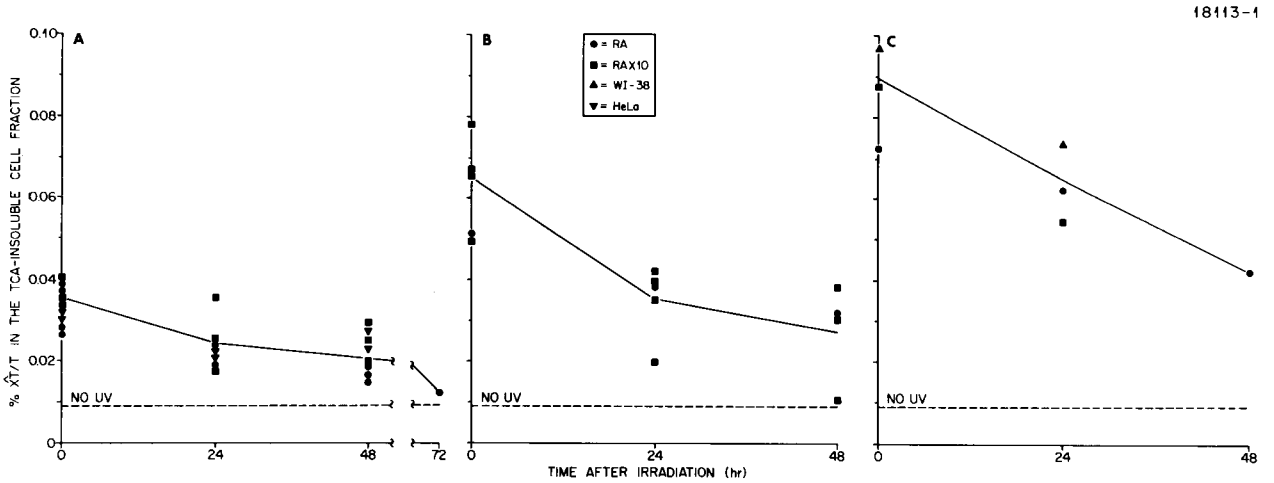

Fig. 7.3.1. Loss of Pyrimidine Dimers from the TCA-Insoluble Cell Fraction. $A = 50$ ergs/mm^2, $B = 100$ ergs/mm^2, $C = 150$ ergs/mm^2. Points shown in A are experimental values. Lines connect mean values for each cell line. In B and C lines connect values of individual experiments.

2. Dimer loss from the TCA-insoluble cell fraction is accompanied by the appearance of dimers in the TCA-soluble cell fraction.
3. Dimer excision is not an exclusive property of heteroploid established cell lines, since it also occurs in the human diploid cell strain WI-38.
4. Of the total dimers induced, about 30% are cytosine-thymine dimers, which are also excised at roughly the same rate as thymine-thymine dimers.
5. The excision seen in these experiments is not an artifact of label conservation in thymine but not dimer because the addition of 10^{-5} M nonradioactive thymidine has no effect on excision (Table 7.3.1).
6. Semiconservative DNA synthesis is not required for the excision process (although some amount of "repair replication" may occur) since hydroxyurea at 2.5 mM concentration did not affect excision (Table 7.3.1).
7. Although caffeine at relatively high concentrations can inhibit excision in bacteria, at 9×10^{-3} M, caffeine had no effect on human cell dimer excision (Table 7.3.1).

These observations seem to suggest that an excision-type dark-repair system perhaps similar to that found in uv-resistant bacteria may occur in human cells. We are unable to account for the fact that only about 50% of the dimers are lost in the first 24 hr with little or no loss thereafter. It is, however, noteworthy that other authors have reported a similar pattern of "repair" of lesions induced in HeLa cells by alkylating agents.[6]

Table 7.3.1. Effect of Several Agents on Loss of Dimers from UV-Irradiated RAX10 Cells

Dose = 150 ergs/mm^2

	Percent of Thymine as Dimer	
	0 hr	24 hr
Control	0.082	0.047
Thymidine (1×10^{-5} M)	0.101	0.048
Caffeine (9×10^{-3} M)	0.078	0.039
Hydroxyurea (2.5×10^{-3} M)	0.066	0.038
Average	0.092	0.043

All agents are final concentrations in the medium and were added to cultures immediately after irradiation. Values are not corrected for radioactivity in the dimer region of unirradiated cells (0.009%).

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7.4 THE EFFECT OF TRITIATED THYMIDINE ON SISTER CHROMATID EXCHANGE IN A RING CHROMOSOME

J. G. Brewen[1] W. J. Peacock[2]

Studies on the segregation of tritiated thymidine among sister chromatids at successive mitotic divisions following incorporation of the isotope into chromosomal deoxyribonucleic acid have revealed a high frequency of sister chromatid exchange.[3] It was originally concluded by Taylor[3] that these exchanges occurred spontaneously, and this conclusion was supported by the work of Marin and Prescott.[4]

In recent years, evidence[5] and hypotheses[6] have been presented that indicate sister chromatid exchanges are, for the most part, radiation induced. Olivieri and Brewen's[5] evidence, however, was obtained in an experimental system analogous but not identical to the condition of paired sister chromatids.

Venous blood was obtained from a patient who carried a ring chromosome of the D group in over 95% of his circulating leukocytes. Standard blood cultures were made in duplicate. One of the two cultures was pulse labeled with tritiated thymidine for 6 hr; the other culture served as a control. The pulse was given 30 to 36 hr after culture initiation. Beginning 48 hr after culture initiation, aliquots from each culture were fixed at 6-hr intervals following a 6-hr colchicine accumulation of metaphase and C-anaphase figures. The cells were then analyzed for ring chromosome presence and appearance and the tritium-treated cells subsequently analyzed for presence of the isotope.

The occurrence of an odd number of sister chromatid exchanges in a ring chromosome will produce a dicentric ring chromosome that is easily identified in a C-anaphase figure. Tables 7.4.1 and 7.4.2 summarize the results of dicentric ring formation in tritium-treated and control cultures. It is clear that tritium results in a 2 to 3 times higher frequency of sister chromatid exchange than normally occurs in a ring chromosome. It is also evident that there is no dose effect, as both first and second postlabeling divisions have the same yield of exchange, although there is a one-half reduction in dose in second-division cells.

Analysis of ring loss in both cultures shows that there is a higher rate of loss in tritium-treated cultures. The increase in ring loss is of the same magnitude as the increase of dicentric ring formation, as expected.

It was also noted that the frequency of sister chromatid exchange in the rod chromosomes was the same as the frequency of *induced* dicentric formation.

The two- to threefold increase in dicentric ring frequency in the tritium-treated cells indicates that a significant portion of sister chromatid exchanges are radiation induced. There is no direct way of measuring the spontaneous frequency of exchange in the rod chromosomes, but it might be expected to be considerably less than in the ring because of a ring chromosome's instability.

Table 7.4.1. Frequency of Dicentric Rings in C-Anaphase Figures of the Control Culture

Fixation Time (hr)	No. Cells Scored	Percent Dicentrics
48	107	12.1
60	121	12.4
72	225	11.6
Total	453	11.9

Table 7.4.2. Frequency of Dicentric Rings in C-Anaphase Figures of Tritium-Labeled Cultures

Division No.	No. Cells	Percent Dicentrics	Percent SCE[a]
First	215	30.2	
Second	223	26.0	27.7

[a]SCE = sister chromatid exchange frequency in rod chromosomes.

The lack of a dose effect is consistent with all the other published data on the question of the origin of the exchanges. This lack of dose affecting exchange yield makes Wolff's hypothesis[6] suspect, although his conclusions were correct.

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[1]USPHS Postdoctoral Fellow at Division of Plant Industry, CSIRO, Canberra, Australia, during the course of this work.

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7.5 CHROMOSOME ABERRATIONS AND THE EXCHANGE HYPOTHESIS

J. G. Brewen[1] R. D. Brock[2]

At present there are two hypotheses as to the mechanism of chromosome aberration production. The older is the "breakage-first" hypothesis, and its name is self-explanatory. A more recent hypothesis is that of Revell,[3] or the "exchange" hypothesis. Whereas the "breakage-first" hypothesis dictates the initial aberration-forming event is the physical breaking of a chromosome, the exchange hypothesis says it is exchange initiation. From this it follows that all aberrations are exchanges, and since an exchange necessitates two "lesions" or breaks in order to occur, it also follows that the dose kinetics of the deletion class of aberration are expected to be different according to the two hypotheses.

All attempts at clarifying this issue have utilized chromatid-type aberrations, a type especially unsuitable to dose relation studies. We have made a study on the dose characteristics and fractionation effect of chromosome-type deletions. Chromosome-type aberrations are superior to chromatid-type because there are no problems of differential stage sensitivity and mitotic delay.

Whole blood of a male wallaby (*W. bicolor*, $2n = 11$) was irradiated and cultured in the usual way. Two dose curves were done, as well as a two-time fractionation experiment.

Metaphase chromosomes were accumulated with colchicine, and slides made in the manner described elsewhere. The metaphase figures were then analyzed for chromosome-type aberrations.

The doses and fractionation intervals employed are adequately summarized in Table 7.5.1 and Fig. 7.5.1.

A least-squares analysis of the regression of aberration frequency plotted against dose shows

Table 7.5.1. Aberration Frequencies and Standard Errors Obtained in Fractionation Experiment

500 Cells scored at each treatment

Dose (rads)	Time Dose Given (hr)	Fractionation Interval	Percent Centric Rings + Dicentrics	Percent Acentric Rings + ID	Percent Terminals
150	0		6.80 ± 1.17	10.20 ± 1.43	11.60 ± 1.52
150	3		11.80 ± 1.54	9.60 ± 1.39	10.80 ± 1.47
300	0		28.40 ± 2.38	32.80 ± 2.48	28.60 ± 2.39
300	3		33.40 ± 2.58	34.80 ± 2.64	29.60 ± 2.43
150 + 150		15 min	28.20 ± 2.37	31.80 ± 2.52	28.00 ± 2.37
150 + 150		3 hr	19.00 ± 1.95	23.60 ± 2.17	21.00 ± 2.05
Expected yield with interaction[a]			30.90 ± 2.49	33.80 ± 2.60	29.10 ± 2.45
Expected yield with additivity[b]			18.60 ± 1.36	19.80 ± 1.41	22.40 ± 1.49

[a]Mean of the two 300-rad doses.

[b]Sum of the two 150-rad doses.

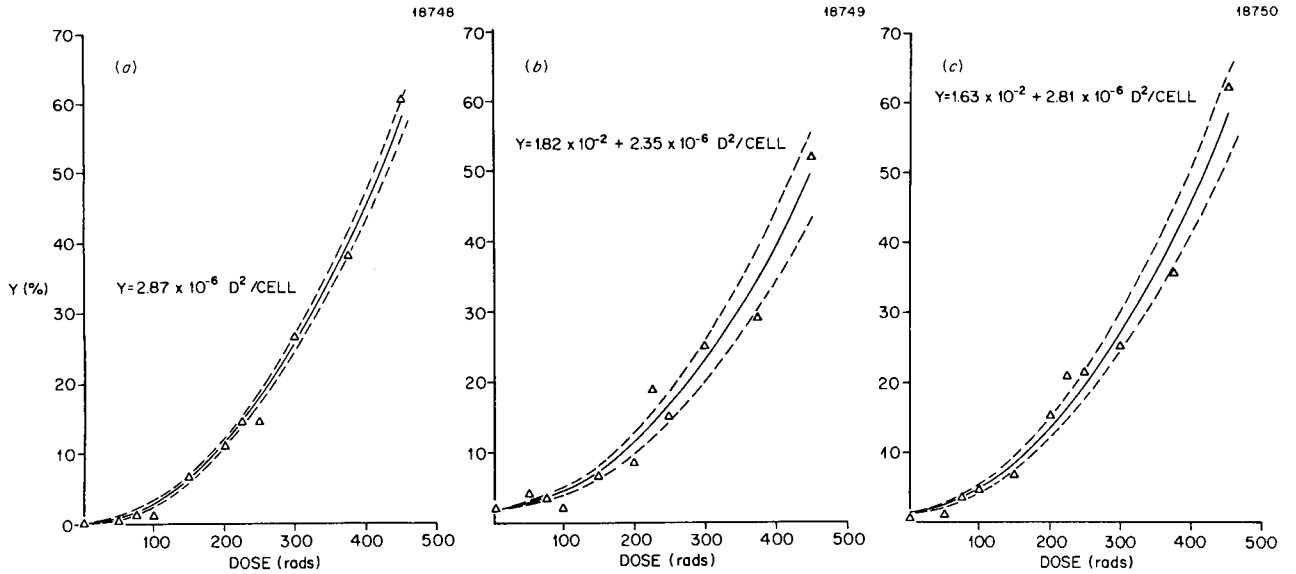

Fig. 7.5.1. Observed Aberration Frequencies and Best-Fitting Model. The dashed lines indicate the standard error of the best fit. (*a*) Centric rings plus dicentrics. (*b*) Acentric rings plus intercalary deletions. (*c*) Terminal deletions.

all aberrations to best fit a dose-square model. The fits are shown in Figs. 7.5.1*a*, *b*, and *c*. Terminal chromosome deletions also responded to a fractionation effect, as indicated by the data in Table 7.5.1.

An analysis of over 100 dicentrics showed 11% to be unequivocally incomplete in their rejoining.

The predominant two-track dose curve and fractionation effect for terminal deletions indicate that many of them result from two independent ionizing events and therefore could conceivably be the result of incomplete exchange. Calculations have been made on the basis of the total number of complete exchanges observed that show all terminal deletions could result from incomplete exchange if 15 to 25% of all exchanges were incomplete. The observed value of 11% of incompleteness in the dicentric class is not significantly different from the predicted value.

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[1]USPHS Postdoctoral Fellow at Division of Plant Industry, CSIRO, Canberra, A.C.T., Australia, during the course of this work.

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8. Mammalian Genetics

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8.1 THE FREQUENCIES OF X-CHROMOSOME LOSS IN FEMALE MICE EXPOSED TO DOSES OF 50 AND 400 r OF X RAYS

W. L. Russell Patricia R. Hunsicker
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The experiment to be described was undertaken to test whether two of the factors that markedly affect the frequency of radiation-induced mutations at specific loci would have a similar influence on a different type of mutational damage. The two effects on specific-locus mutation induction that suggested this additional test were, first, the dependence of mutation frequency on the interval between irradiation and conception, and, second, the departure from linearity of the dose-mutation curve at low doses.

In female mice, it has been shown, first with neutrons[1] and then with x rays,[2] that, as the interval between irradiation and conception extends beyond six or seven weeks, the mutation frequency in the offspring plunges to an extremely low level. This finding has direct application to the estimation of genetic hazards and is of basic importance in our understanding of the mutation process and the factors that affect it. It was clear that these aspects of the phenomenon could be explored further by testing the effect of the irradiation-conception interval on mutational events other than the specific-locus mutations used in the original finding. We chose a type of mutational damage quite different from that detected by the specific-locus method, namely, the induction by x rays of X-chromosome loss as a result of chromosome breakage.

Since the x-ray dose had to be low in order to avoid the early loss of fertility that occurs with high doses, the experiment could easily be designed for the second objective by simply running a small part of it at a high dose. Although the females in this portion of the experiment would lose their fertility after bearing only one or two litters, the mutation frequency could be compared with that from the early matings of females exposed to the low dose. In the x-ray experiment on specific loci, it had been found that a 50-r dose to females gave a much lower mutation frequency than expected, on a linear basis, from the mutation frequency at 400 r.[3] This was taken as evidence that some repair of mutational or premutational damage can occur at low doses as well as at low dose rates. It was of considerable interest to find out whether the same phenomenon would be observed with X-chromosome losses.

Mature F_1 hybrid female mice from the cross of 101 and C3H strains were exposed to 50 or 400 r of approximately 80 r/min x irradiation and mated, 24 hr later, with males carrying either one of two sex-linked genes, Tabby (*Ta*) or Greasy (*Gs*), predominantly the latter. The offspring were scored for occurrences of presumed *Ta*/0 or *Gs*/0 females, as well as for any other detectable departures from the *Ta*/+ or *Gs*/+ phenotype for females and wild-type phenotype for males. The presumed *Ta*/0 and *Gs*/0 females, as well as other exceptional animals, were checked by breeding tests and chromosome counts.

By both genetic and cytological criteria, all presumed *Gs*/0 or *Ta*/0 animals tested to date appear to be X-monosomic. On the simplest assumption, the X-chromosome loss could be the result either of breakage or of nondisjunction. The latter would also produce X^MX^MY and $X^MX^MX^P$ exceptions, phenotypically nondetectable with the markers in this experiment. However, independent data[4] indicate that induced X^MX^MY must be rare. It is, therefore, at least provisionally assumed that most, if not all, *Gs*/0 or *Ta*/0 exceptions in the present experiment were the result of x-ray-induced chromosome breakage. No unirradiated controls were used, since females in normal stocks with the requisite sex-linked markers had shown no spontaneous occurrences of X-chromosome loss in 2576 offspring.[4,5,6]

The experiment has not yet proceeded far enough to provide adequate data on the effect of the interval between irradiation and conception on X/0 frequency. Data on the second objective of the experiment, the effect of a low dose, are, however, already informative. The results are shown in Table 8.1.1. The observed frequencies of 10 and 15 X-chromosome losses in the female germ cells exposed to, respectively, 50 and 400 r are very close to the 9.9 and 15.1 expected on the basis of a straight-line relation between mutation frequency and dose. Thus the data provide no evidence for dose-dependent repair of the kind of damage, presumably chromosome breakage, that leads to X-chromosome loss. It would appear that the hazard from this type of genetic damage, unlike that from mutations scored by the specific-locus method, remains linearly related to dose as far down as 50 r.

Table 8.1.1. Maternal X Chromosome Losses in Offspring Conceived in the First Six Weeks After Irradiation of Wild-Type Female Mice Mated to *Ta*/Y or *Gs*/Y Males

Dose (r)	*Ta* or *Gs* / + ♀♀	*Ta* or *Gs* / 0 ♀♀	
		Observed	Expected[a]
50	3753	10	9.9
400	699	15	15.1

[a]On the assumption of a straight-line relation between mutation frequency and dose.

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8.2 COMPARATIVE STUDY OF FIVE X-AUTOSOME TRANSLOCATIONS INVOLVING LG1 IN THE MOUSE

L. B. Russell Clyde S. Montgomery

A large part of the evidence both for the development[1,2] and later modification[3,4] of the single active X-chromosome hypothesis has come from X-autosome translocations in the mouse,[5–7] and, in particular, from five independent T(X;1)'s, discovered at this laboratory, whose properties can be compared. Because of considerable fertility problems, a detailed study of these has taken several years, although the broad features of the results have been used earlier to develop hypotheses of X-chromosome activity.[3,4,7] Results of the detailed comparative study of T(X;1)'s, which has now been completed, will be summarized here.

The rearrangements named R2, R3, R4, R5, and R6 were found in the course of specific-locus mutation-rate experiments. Four of them came from postgonial irradiated stages; one, R6, from a control mating. The results to be summarized come from almost 200 different types of crosses involving these rearrangements. The letter R will be used to indicate presence of a heterozygous T(X;1) and also to designate location of breakpoint. Gene symbols shown in parentheses will indicate location of these alleles in a translocated chromosome.

Viability. – Two measures of viability were used. Survival to seven days was measured in crosses in which the R animals are phenotypically distinguishable and where the segregating markers used do not present known viability problems. Among 2034 classified males, there was no evidence of reduced survival in any of the R stocks. The frequency of translocation females at seven days of age was, however, less than 50% in all stocks (a total of 3064 females classified), the difference being highly significant in four of the five comparisons. A second measure of viability, applied to females set up for breeding, was young-adult death and sterility (the latter, for various reasons, probably due to lack of general well-being, rather than to specific reproductive deficiency). The incidence of these parameters was higher for R than for non-R females in all stocks (totals of 866 and 284, respectively), and the comparison for sterility alone, 8.2 vs 2.5%, was quite significant.

Weight. – Presence of all T(X;1)'s has a marked effect on body size in females, for which there was a weight reduction of from 11 to 26% in different stocks. There was no effect on males.

Fertility. – In extensive fertility tests of 277 males, R males were found to be invariably sterile, even though mated from less than six weeks of age on. Testis weights of 97 R and 61 non-R males showed no overlap, the former falling entirely within the range of from 20 to 80, the latter from 140 to 270 mg.

Average litter sizes of R females (based on a total of 8104 offspring) are only 38% of those produced by contemporary non-R segregants (based on 9425 offspring), even though the former averaged a smaller number of litters. For individual T(X;1) stocks, the ratios vary from 35.1 to 43.8%. In three of the stocks, there is no overlap at all in ranges of R and non-R average litter sizes.

The fact that the ratios are markedly below 50% could indicate either that R females are poor mothers or, alternatively, that unbalanced segregations are frequent. Random 2:2 segregation from the association of four chromosomes would theoretically yield 33% normal litter size, but the actual ratio might be slightly higher due to better survival of embryos in small litters.

Segregation. – It is obvious from the small litter sizes that segregants with autosomal imbalance do not survive, and this can also be shown by the phenotype when appropriate markers are used.

Imbalances involving an entire X chromosome are viable in the translocation stocks. R females that have failed to receive a paternal sex chromosome are self, resembling R males; R males with an extra X are variegated, similar to R females (these findings were basic to the formulation of single-active-X hypothesis). The frequency of the latter type was between 0.11 and 0.16 (based on a sample of 1849 males). The reported spontaneous incidence of paternal nondisjunction is 0.02%. It may be assumed that the excess in R stocks is due to 3:1 segregation from the association of four chromosomes in the translocation mother. The complementary type, however, that is, daughters receiving the intact autosome but lacking the intact X, is probably at least one order of magnitude more frequent. This would indicate that X loss in R females can occur independently of 3:1 segregation. It should be noted that spontaneous loss of X^M is extremely rare.[1]

Inactivation of Autosomal Loci. – Whether or not a given autosomal locus is inactivated at all by a given rearrangement and, if so, how much, can be revealed by introducing an intact autosome carrying a lower allele at that locus. If the locus is subject to inactivation, this procedure will result in variegated females mosaic for (1) cells in which the given autosomal gene in the rearranged chromosome is suppressed ("uncovering" the lower allele in the intact chromosome) and (2) cells in which the rearranged chromosome is active. The variegating phenotype, which could theoretically be either that of the hemizygous lower allele or that of some compound, indicates whether inactivation is to a nullo state or to some intermediate level. Frequency of inactivation is indicated by the average proportion of the body having the variegating phenotype.

Inactivation has been tested for the following autosomal loci: c, *p*, *tp*, *pu* [(c-(3)-*tp*-(13)-*p*-(22)-*pu*)]. For the c locus, full-frequency inactivation (i.e., 50% of body having the recessive phenotype) is produced by R3; somewhat less by R6; about half frequency by R4; less than half by R5; and none whatsoever by R2. For the *p* locus, R6 and R5 give full and half frequency, respectively; R2, about one-quarter; R3 and R4 barely any. The *tp* locus, difficult to study quantitatively, is subject to inactivation in all the rearrangements, but is probably most frequently affected in R2. The *pu* locus (affecting tail development) has been studied only in R5 and R6, where it is either not inactivated or not inactivated with a high enough frequency to affect the total phenotype.

Inactivation is to a nullo form, even in such rearrangements in which the given locus is affected only half or less than half of the time. Since the allele in the intact chromosome is therefore expressed in hemizygous condition, it is possible to determine whether it is an amorph or a hypomorph. It has been concluded that *p* is the latter, since (*p*)/*p* is variegated, that is, 0/*p* and *p*/*p* are distinguishable.

Location of Autosomal Breakpoints. – The LG1 positions of rearrangement points, R's, relative to c and *p* loci have been determined in three-point crosses. R4 and probably R3 lie outside the c-*p* interval, on the c side; R5 and R6 lie outside on the *p* side; and R2 lies within the interval, probably between c and *tp*. Because R2 is broken close to c yet does not inactivate it, special efforts were made to check for other possible types of rearrangements, namely *non*reciprocal translocation and insertion. These could be rejected on the basis of genetic and cytological results too complex to describe here.

Recombination values, based on sample sizes ranging from 346 to 1369, are as follows. R2: c-R = 0, R-*p* = 9.7; R3: R-c = 0.2, c-*p* = 16.6; R4: R-c = 6.9, c-*p* = 19.6; R5: *p*-R = 8.1, c-*p* = 19.4; R6: *p*-R = 2.2, c-*p* = 21.6. In cases where the autosomal breakpoint lies outside the c-*p* region (R3, R4, R5, R6), recombination between these two loci is not reduced and may even be slightly enhanced. There is also evidence (R2) for moderate crossover inhibition in the immediate vicinity of the breakpoint.

The relation of breakpoint location to relative inactivation of the various autosomal loci led to our idea that the mouse X chromosome bears a center from which inactivating influences can emanate along gradients.[3] The inactivation

center is inoperative when only one X is present; with two or more present, it is inoperative in one of them, which differentiates at random early in embryology. In the case of an X-autosome translocation, part of the autosomal segment that has been joined to that portion of the X that bears the inactivation center also comes under the influence of this center. Preliminary mapping of X breakpoints has further confirmed our views and has put limits on the location of the inactivation center.

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8.3 RNA SYNTHESIS IN MOUSE OOCYTES

E. F. Oakberg Roselynn A. Dynesius
Evelyn C. Lorenz Diane W. Slover

Previous experiments revealed that the rate of incorporation of ^{3}H-uridine varied with stage of development of the mouse oocyte.[1] These experiments have been repeated in order to obtain more data and to evaluate the effect of RNase digestion on ^{3}H-uridine-labeled oocytes.

Ovaries were fixed in acetic alcohol (3:1), sectioned at 3 μ, deparaffinized, and hydrated. Half of the slides from each mouse then were placed in a solution of 1 mg RNase (bovine pancreas salt-free 5 λ cryst.) per milliliter in distilled water at 37°C for 1 hr 50 min, rinsed in distilled water, placed in 5% cold (0°C) TCA for 1 hr, rinsed in distilled water, rinsed thoroughly in 70% ethyl alcohol, and placed in distilled water. All slides were then air-dried and coated with Kodak NTB_2 liquid emulsion diluted 1:1 with a 0.1% solution of Dreft.

^{3}H-uridine labeling was effectively removed by the RNase plus cold TCA treatment. Analysis of various cell RNA species was not done, but it is probable that the ^{3}H-uridine incorporation observed in the mouse oocytes represents synthesis of ribosomal and messenger RNA.

Data on oocyte labeling in the slides which were not treated with RNase were comparable with those obtained in the earlier experiments and confirm our conclusion that active RNA synthesis occurs during growth of the mouse oocyte but not in oocytes contained in follicles with an antrum.

No specific relationship between ^{3}H-uridine metabolism and repair of radiation damage has been demonstrated, but the changing pattern of ^{3}H-uridine incorporation with growth of the oocyte may indicate general levels of metabolic activity which could be important in response of the oocyte both to radiation-induced cell killing and to mutation.

Reference

[1]E. F. Oakberg *et al.*, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, pp. 115–16.

8.4 STEM-CELL RENEWAL IN THE SEMINIFEROUS EPITHELIUM OF THE MOUSE

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Type A spermatogonia are the stem cells of the seminiferous epithelium, and, by the process of stem-cell renewal, maintain their own numbers, yet give rise to a constant supply of differentiated germ cells. The exact mechanism by which the stem-cell population is maintained, however, is not well understood.

Recently, Clermont and Bustos[1] have presented evidence in the rat for a spermatogonial type which they have designated A_0. These cells are thought to divide at a slow rate and out of synchrony with the majority of spermatogonia. The function of these cells is not clear, but they are thought to be "reserve" stem cells available in case of depletion of A gonia; as, for instance, in the irradiated testis. The presence of this cell type in the mouse, therefore, could change our previous interpretations of both histological and genetic effects of radiation on spermatogonia.

The duration of the cell cycle of types A_2–A_4 spermatogonia of the mouse is 26 to 30 hr, with

an average DNA synthetic time of 6 to 7 hr.[2] It should be possible to demonstrate the existence of A_0 spermatogonia by their failure to label if ^{3}H-thymidine is present throughout one complete cell cycle. Accordingly, 101 × C3H hybrid male mice were given six injections of 12.5 μc of ^{3}H-thymidine (specific activity 1.9 curies/millimole) at 5-hr intervals. Autoradiographs were prepared, and 1000 type A spermatogonia were scored for each mouse.

Data on frequency of labeled A spermatogonia 1 hr after the sixth injection are given for the 12 stages of the cycle of the seminiferous epithelium in Table 8.4.1. The low incidence of labeling at stages IV–VII, when the A_1 spermatogonia are mitotically inactive, was expected. It also is apparent that a smaller proportion of the A_1 and A_2 population is dividing at stages IX–XII (~75% labeling) than is the case for A_3 and A_4 in stages I–II (~90% labeling). At all of these times, however, both labeled and unlabeled cells appear (from data not given here) to divide at approximately the same relative frequency. Thus either some cells were not labeled by our techniques or some cells have an unusually long G_2 period.

Examination of the data for the 1–15 day interval reveals that about half of the mitotically inactive A spermatogonia at stages III–VIII are derived from cells which were labeled at stages XII–II, that is, in the previous one or two mitotic divisions. The fact that 90% of the spermatogonia labeled at stages I–II, together with divisions among the remaining 10% of unlabeled cells, would suggest that all A spermatogonia divide at least once during this interval. This makes untenable the hypothesis that the stem cells are differentiated in the divisions at IX and do not divide again until stage IX of the following cycle.[2]

Division of all A spermatogonia at stages I–II also excludes the existence of a spermatogonial type (A_0?) which does not regularly participate in spermatogonial renewal in the mouse.

Division of some A spermatogonia, however, is quite irregular. This is indicated by the lower labeling observed at stages IX–XII in comparison with that observed at stages I–II and by the presence of some heavily labeled cells ten days after ^{3}H-thymidine injection. By ten days, all A spermatogonia have had an opportunity to divide four times, and some have passed through five mitotic peaks. If all cells had divided at each mitotic peak, the label would have been diluted below recognizable levels. Eventually, however, these cells do divide and produce differentiated cells, for at 15 days no heavily labeled A spermatogonia are present, and some lightly labeled, recently differentiated intermediate spermatogonia can be seen.

Thus there is no evidence for the existence of an A_0 (reserve stem cell) spermatogonial population in the mouse. Some type A spermatogonia, however, do not divide regularly, but they all eventually participate in stem-cell renewal.

Table 8.4.1. Frequency of Labeled Type A Spermatogonia at Each of the 12 States of the Cycle of the Seminiferous Epithelium of the Mouse 1 hr After Six Injections of ^{3}H-Thymidine

Stage	Total Cells	Proportion Labeled
I	148	0.892
II	172	0.890
III	14	0.214
IV	21	0.095
V	21	0.048
VI	62	0.032
VII	71	0.028
VIII	51	0.275
IX	103	0.660
X	37	0.757
XI	131	0.748
XII	169	0.781

References

[1]Y. Clermont and E. Bustos, *Anat. Record* **154**, 332 (1966).

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8.5 TRANSMISSION OF RADIATION-INDUCED DOMINANT SKELETAL MUTATIONS IN MICE

U. H. Ehling

Our published estimate for dominant mutations affecting the skeleton after single exposure with

600 r is 2.9×10^{-5} mutation gamete^{-1} r^{-1} for postspermatogonial stages and 1.1×10^{-5} mutation gamete^{-1} r^{-1} for spermatogonia. The 95% confidence intervals are 1.6 to 5.2 and 0.4 to 2.5 respectively.[1] The figures were based on the relatively frequent occurrence of a certain class of abnormalities in the offspring of irradiated males and the almost complete absence of such abnormalities in the offspring of control males. Although it would have been desirable to test the mutational origin of at least a sample of the individual abnormalities by carrying out breeding experiments, this was not feasible in the original experiment, where the first-generation offspring were sacrificed at 26 to 28 days, that is, before they reached the reproductive age.

The present experiment was designed specifically to permit breeding tests on a sample of presumed mutations. The first-generation offspring are not sacrificed until they have produced one litter. Sacrificed animals are processed for skeletal study by a modification of Dawson's technique. The whole skeletons are examined in detail in Alizarine Red S preparations. Offspring of first-generation animals that were classified as presumed dominant mutations affecting the skeleton are test bred. Offspring from animals classified as nonmutant are discarded.

To date, three dominant mutations affecting the skeleton were found to be transmitted to the second and later generations. One of these mutants was found in an earlier experiment where the offspring were derived from sperm irradiated as spermatogonia.[1] Two of these mutants were found in this experiment where the offspring were derived from sperm irradiated as spermatozoa or spermatids. One mutant was found in a group of 108 offspring which were produced by males irradiated with 600 r. The other mutant was found in a group of 86 offspring produced by males which were irradiated with 100 r followed by 500 r 24 hr later. The test of two additional presumed mutations is incomplete.

The evidence that a sizable proportion of the presumed dominant mutations are due to gene mutations was discussed earlier.[1] In the discussion it was emphasized that it might be possible to recover some of the presumed mutations. The results of this experiment demonstrate that it is feasible.

Reference

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8.6 TOXICITY, FERTILITY, AND DOMINANT LETHAL MUTATIONS AFTER INJECTION OF IRRADIATED SUCROSE IN MICE

U. H. Ehling
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Sandra W. Huff

The induction of dominant lethal mutations in mice with x rays[2] or alkylating agents[3] is due to chromosomal aberrations. The observation that irradiated sucrose induced chromosomal aberrations in human lymphocyte cultures[4] made it mandatory to test this substance for the induction of dominant lethal mutations in mice.

A 2% solution of reagent-grade sucrose in double-distilled water was irradiated at 25°C in a cobalt source at approximately 6800 rads/min for a total of 2×10^6 rads. The solution was adjusted to pH 7.0 with NaOH and concentrated in vacuo at 25°C to a thick syrup in a flash evaporator. The syrup was dissolved in distilled water, adjusted to pH 7.0 with NaOH, and diluted to a final concentration of 20%. The 20% solution was either sterilized by autoclaving for 20 min at 15 lb pressure or by passing through a Millipore filter without autoclaving. A similar process of concentration was used to prepare the unirradiated 20% sucrose control solution.

The three types of differently treated sucrose solutions were injected intraperitoneally into 71 hybrid $(101 \times C3H)F_1$ male mice, 10 to 12 weeks old. In one experiment ten males were injected with 1 cm^3 of Hank's balanced salt solution. Immediately after injection, each male was caged separately with two virgin hybrid females, also 10 to 12 weeks old. A second and third mating with two females was made after 7 and 14 days respectively. The uterine contents of fertilized females were examined 14 to 17 days after conception. Single pair matings were made 22 to 120 days post treatment. Litters were recorded and killed at birth. With our mating scheme, 1- to 7-day postinjection sperm are treated as spermatozoa of vas and epididymis, 8- to 14-day postinjection sperm as testicular sperm and late spermatids, 15- to 21-day postinjection sperm as early spermatids, and 22- to 35-day postinjection sperm as spermatocytes. Gametes treated as spermatogonia are utilized six weeks after injection.[5]

Experiments performed in the Biology Division demonstrated that irradiated, autoclaved sucrose

produces large numbers of chromosomal aberrations in human lymphocyte cultures, but neither irradiation nor autoclaving by itself produces any detectable effect.[6] Based on these observations we used in the first experiment for the calculation of the yield of dominant lethal mutations as the control group the males treated with irradiated sucrose; in the second experiment the males of the control group were treated with a Hank's balanced salt solution.

An injection of 1.5 cm^3 of an irradiated and autoclaved sucrose solution was lethal to all 25 animals tested. The injection of the same amount of irradiated or autoclaved sucrose solution was lethal to, respectively, 1 out of 10 and 2 out of 10 of the treated males (Table 8.6.1). Deaths in all groups occurred within three days.

In both experiments the percentage of fertile matings 1 to 7 days post injection of irradiated, autoclaved sucrose solution is below the frequency of the control group (Table 8.6.2). The decrease in matings is probably due to the stress of the injection of irradiated, autoclaved sucrose solution (Table 8.6.1).

The frequency of dominant lethal mutations is summarized in Table 8.6.2. The statistically insignificant deviations from zero for the calculated values of dominant lethal mutations indicate that the treatment was ineffective in inducing dominant lethal mutations in spermatozoa and spermatids of mice.

The maximum number of litters produced by a pair of mice in the 22- to 120-day interval post injection is 5. The average numbers of litters produced in this interval for the three groups of the first experiment are as follows: irradiated sucrose group 4.8; autoclaved sucrose group 5.0; and irradiated, autoclaved sucrose group 4.7. The pooled frequency of 46 males treated with Hank's balanced salt solution in the same interval but at different times was 4.7 litters per pair. These differences are clearly not significant. The differences in the number of offspring per pair in the three groups treated with sucrose are also insignificant. From these data it can be predicted that the induction of dominant lethal mutations in spermatocytes is unlikely. The reproductive performance of the males, when treated spermatogonia were utilized, gives no indication that the development of this cell stage was impaired by the treatment with a 20% irradiated, autoclaved sucrose solution.

Table 8.6.1. Toxicity of 20% Sucrose Solution in (101 × C3H)F_1 Male Mice

Experiment	Treatment of Solution	Volume (cm^3)	No. of Males	Survival (%)
First	Irradiated	1.5	10	90
	Autoclaved	1.5	10	80
	Irradiated and	1.5	20	0
	autoclaved	1.0	25	100
Second	Irradiated	1.0	15	100
	Irradiated and	1.5	5	0
	autoclaved	1.0	15	93

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8.7 ^{3}H-URIDINE INCORPORATION INTO LM CELLS IN VIVO AND IN VITRO AND INTO NORMAL MOUSE TISSUES

R. B. Cumming

It has been shown that LM cells, when injected into suitably treated C3H mice at a dose of 1×10^7 cells per animal, produce large, rapidly growing, nonmetastasizing fibrosarcomas in nearly 100% of the animals so treated. While LM-cell-induced tumor incidence is the same in male and female mice, the regression rate is significantly higher in males.[1]

Table 8.6.2. Ineffectiveness of 20% Sucrose Solution to Induce Dominant Lethal Mutations in Spermatozoa and Spermatids of Mice

Experiment	Treatment of 20% Sucrose Solution	Volume (cm^3)	Mating Post Injection (days)	No. of Females	Fertile Matings (%)	No. of Implants	No. of Embryos	Dead Implants (%)	Frequency of Dominant Lethal Mutations (%)[a]
First	Irradiated[b]	1.5	1–21	54	85.2	329	312	5.2	0
	Autoclaved	1.5	1–7	16	81.3	96	86	10.4	2.5
			8–14	16	93.8	110	101	8.2	0.7
			15–21	16	100.0	112	106	5.4	2.3
	Irradiated and autoclaved	1.0	1–7	50	62.0	208	190	8.7	9.6
			8–14	50	60.0	217	197	9.2	3.2
			15–21	50	76.0	279	249	10.8	3.4
Second	Hank's balanced salt solution[b]	1.0	1–21	60	88.3	376	337	10.4	0
	Irradiated	1.0	1–7	30	70.0	143	135	5.6	−1.1
			8–14	30	73.3	162	141	13.0	−0.8
			15–21	30	90.0	194	165	14.9	3.9
	Irradiated and autoclaved	1.0	1–7	28	67.9	141	136	3.5	−12.6
			8–14	28	85.7	172	155	9.9	−1.6
			15–21	26	88.5	164	141	14.0	3.6

[a] $\left(1 - \frac{\text{embryos experimental group}/♀}{\text{embryos control group}/♀}\right) \times 100.$

[b] Control group.

If tumors are removed from the animals, trypsinized, again grown in vitro, and given a half-hour pulse of ^{3}H-uridine in the first passage after removal, cells from tumors of female mice consistently incorporate more of the isotope than those from tumors of male mice. When material from 15-day-old tumors had been grown in vitro for 48 hr prior to labeling, cells from female tumors showed a 44% higher count rate than cells from male tumors. In 42-day-old tumors the count rate was 54% higher for cells from female tumors than for cells from male tumors.[1]

In preparation for in vivo incorporation studies on this tumor system, an experiment was performed on ^{3}H-uridine incorporation patterns in several tissues of normal eight-week-old male and female C3H mice. ^{3}H-uridine was injected intraperitoneally at 4 mc per kilogram body weight, and tissues were harvested at eight time intervals ranging from 15 min to 24 hr after injection. Data were collected by liquid scintillation counting of TCA-insoluble material from tissue homogenates. Typical data for some tissues 12 hr after injection with ^{3}H-uridine in counts per minute per milligram of tissue, wet weight, follow.

Tissue	♂♂	♀♀
Spleen	641.86	662.60
Liver	278.88	341.66
Kidney	201.22	216.69
Heart	59.68	67.64
Brain	9.29	9.24

No significant differences are noted between the sexes except in the liver, where females incorporate a small but consistent percentage more uridine.

Several experiments have been completed which were designed to test the rate of ^{3}H-uridine incorporation in LM cell tumors in male and female mice. Contrary to the in vitro results, male tumors in vivo incorporate consistently more uridine than female tumors. In an experiment in which male and female mice with LM tumors were injected with ^{3}H-uridine at 4 mc per kilogram body weight, samples of TCA-insoluble material from tumor and liver homogenates were counted by liquid scintillation. The following data are from tissues removed from animals 12 hr after ^{3}H-uridine injection and are expressed as counts per minute per milligram of tissue, wet weight.

Tissue	♂♂	♀♀
Tumor	229.05	174.99
Liver	150.11	215.51

The counts in the liver are down in both sexes compared with animals of a similar age without tumors; however, the relationship of females incorporating more uridine into the lever than males under similar conditions remains. LM cell tumors in male hosts, in contrast, incorporate more ^{3}H-uridine than similar tumors in female hosts.

One hypothesis which could be used to explain the relative differences of activity of LM cell tumors in vivo and in vitro follows: In vivo other tissues, and particularly the liver, compete with the tumor for the available uridine pool. As liver tissue in the female mouse is more active in this competition than in the male, less uridine is available for the female tumor than for that of the male. Competition and hormonal control mechanisms are absent in vitro, where cells from LM tumors from males incorporate uridine at a higher rate than those from females.

Reference

[1]K. B. Hellman *et al.*(in preparation).

9. Mammalian Recovery

C. C Congdon[a]

Secondary Disease (9.1–9.5)

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W. H. McArthur[b]
Roland Eisele[c]
Eiji Endo[c]
Alberta P. Henley
R. E. Toya, Sr.
L. W. Webb[d]
W. R. Conover[e]
Frances E. Hacker[a]

Recovery in Vitro (9.6, 9.7)

L. H. Smith
T. W. McKinley, Jr.
Nancy L. Antosen[f]
Charles L. Paxson[f]

Experimental Hematology (9.8, 9.9)

Joan Wright Goodman
Sarah G. Shinpock
Fay M. Martin[b]
Chareen G. Grubbs

Animal Testing Laboratory (9.10–9.15)

M. L. Simmons[a]
C. B. Richter[a]
R. W. Tennant
D. T. Donald
J. A. Franklin
R. L. Hendren
K. R. Layman
G. L. McNabb
F. E. Meyer
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[d]Loanee
[e]American Cancer Society Postdoctoral Fellow
[f]Student trainee
[g]Temporary

9.1 MORTALITY FROM SECONDARY DISEASE IN RADIATION CHIMERAS

C. C Congdon R. E. Toya, Sr.

The major lines of investigation in our program on treatment of radiation injury have been to study mortality, pathology, immunology, and metabolic alterations in the lethally irradiated animal kept alive by a foreign bone marrow transplant. Although some life-span determination and leukemia prevention work has been done in collaboration with the Pathology and Physiology section of the Biology Division, most of the effort concerns the 90-day period following transplantation. During this interval a large percentage of mice kept alive by the foreign-marrow treatment die of a secondary disease caused by the graft-vs-host reaction. Immunologically competent cells in the donor marrow grow out after transplantation and attack the host.

Some animals recover spontaneously from secondary disease and go on to live in a relatively normal condition for many months.

The observation that spontaneous recovery from the graft-vs-host reaction occurs, formed the basis for a factorial-design–response-surface study of the 90-day mortality problem. With the assistance of the Mathematics Division, Oak Ridge National Laboratory, in planning the experiments and analyzing the data, six experimental designs were carried out.

In the initial five designs the goal was to find the region of the 90-day mortality response surface that gave least mortality. This was essentially completed for allogeneic (homologous) bone marrow donation with the fourth design, and the results are shown in Fig. 9.1.1. For heterologous (rat) marrow transplanted into 950-r irradiated mice, an unusually low-mortality region was found in the fifth design, and further exploration of this region is scheduled.

Surviving animals from these designs were shown to be chimeras in good health with persistent donor marrow grafts. Leukemia induction studies on survivors in certain experimental designs are in progress.

The primary factors under study in these initial designs were age and dose of donor cells, sex of donor and recipient, and day of injection of cells after x ray of the recipient. A sixth design was carried out to find regions of highest mortality in the 90-day mortality response surface varying these same four factors in an allogeneic situation. Overall 90-day mortality in 640 irradiated mice is about 20% in this design, which indicates unusually good recovery from secondary disease rather than the opposite effect which was being sought.

Throughout the factorial design studies we have made the assumption that the host animals have not changed appreciably, but recent observations point to a physiologic change resulting from changes in the laboratory animal environment. Production of mice is now carried out in filter top cages, with absence of the usual mouse parasites and murine viruses. The result is an in-

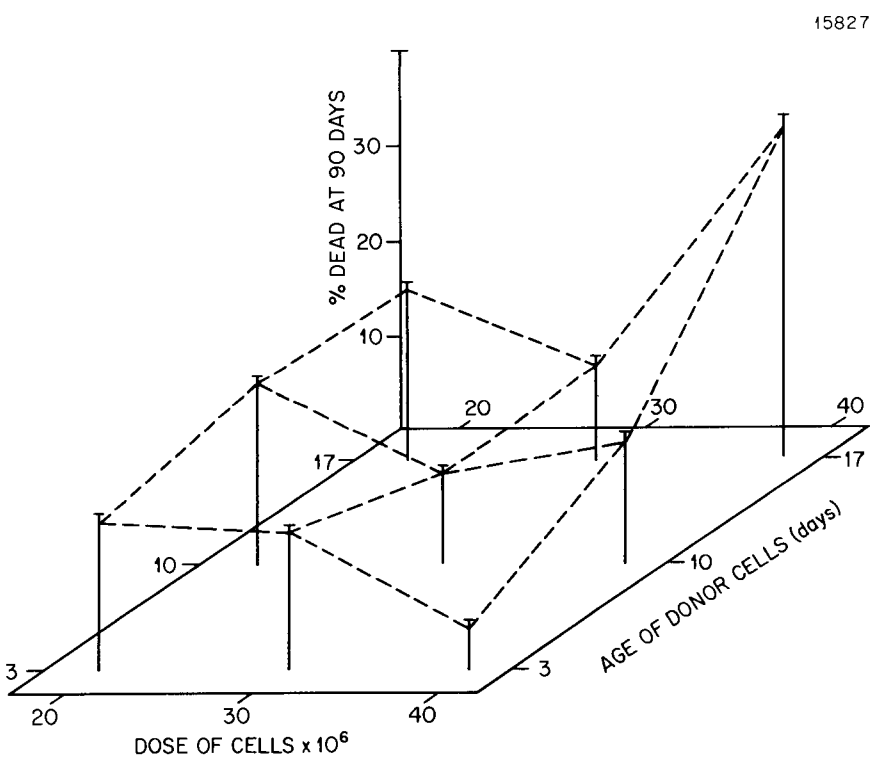

Fig. 9.1.1. Response Surface for 90-Day Mortality when Dose and Age of Foreign Donor Cells Are Varied in Treatment of Lethal X-Ray Exposure.

creasingly hypoplastic lymphatic tissue system in current research animals, compared with that in animals of the original experimental designs.

It seems possible that the reduced lymphatic tissue mass provides less area for graft-vs-host reaction and therefore less mortality. This finding shows a need for adding another factor to the four already involved in our design work – that of the weight of the preexisting lymphatic tissue mass in the lethally irradiated host.

9.2 KIDNEY LYSOZYME IN HYPOXIC RADIATION CHIMERAS

Eiji Endo

Perri *et al.*[1,2] observed that lysozyme activity was elevated in the kidney of rats carrying a nonisogenic tumor. Because the tumor was not genetically identical to the host animal, it seemed possible that immunologic activity of a host-vs-graft type was a factor in producing the increase in the enzyme level. Following up on the work of Perri *et al.*, Suu *et al.* showed that a graft-vs-host reaction in the mouse foreign-marrow radiation chimera also produced a marked increase in kidney lysozyme.[3,4] More recently, Troup and Walford[5] reported that elevated kidney lysozyme is present in neonatal mice with runt disease produced by a graft-vs-host reaction. According to Congdon *et al.*,[6] under extreme hypoxia a graft-vs-host reaction was ineffectual in causing death, and thus one might expect that kidney lysozyme would be lower than in a comparable radiation chimera under the usual atmospheric oxygen levels. Therefore, in the present work, lysozyme was measured in kidneys of mouse foreign-marrow radiation chimeras kept at usual atmospheric conditions and compared with similar chimeras kept under conditions of extreme hypoxia[7] from the time of radiation.

Experimental animals were 12-week-old mice of the $B6C3F_1$/Sim type (C57BL/6 ♀ × C3H/Anf ♂). Donors for isologous bone marrow (IBM) were of the same hybrid type and age. Donors of homologous bone marrow (HBM) were $CD2F_1$/Cum mice (BALB/c Cum ♀ × DBA/2 Cum ♂). The experimental design is shown in Table 9.2.1. All animals were weighed and killed for examination at nine days after irradiation and/or marrow injection. Both kidneys were removed, freed of connective tissue, and weighed before being used in the enzyme assay. Lysozyme was assayed according to the procedure of Suu *et al.*,[3,4] which was based on the method of Shugar.[8]

Table 9.2.1. Experimental Design

Group No.	Treatment	Caging[a]	
		Hypoxia	Standard
1	950 r	+	
2			+
3	950 r + IBM[b] (40×10^6 cells)	+	
4			+
5	950 r + HBM[b] (40×10^6 cells)	+	
6			+
7	Normal	+	
8			+
9	Normal + IBM (40×10^6 cells)	+	
10			+
11	Normal + HBM (40×10^6 cells)	+	
12			+

[a]The mice were kept eight per cage and allowed free access to food and water.

[b]The cells were given intravenously by tail vein within 5 hr after irradiation.

At nine days after irradiation, the kidney lysozyme was markedly elevated in HBM mice, as had been shown in previous studies (Fig. 9.2.1). There was, however, no significant difference between those mice kept under conventional conditions and those kept under extreme hypoxia in the HBM chimera group. In the five different control groups, the enzyme levels were in the same general range, but in all instances the lysozyme measurement was a little higher in the hypoxic situation than under standard caging conditions. The lowest lysozyme level was found with standard caging where no irradiation or marrow injection was given. Next to the HBM chimera group, HBM in unirradiated hypoxic mice give the highest enzyme level.

These preliminary findings did not turn out as expected. If the hypoxia did in fact reduce the graft-vs-host reaction, then kidney lysozyme did

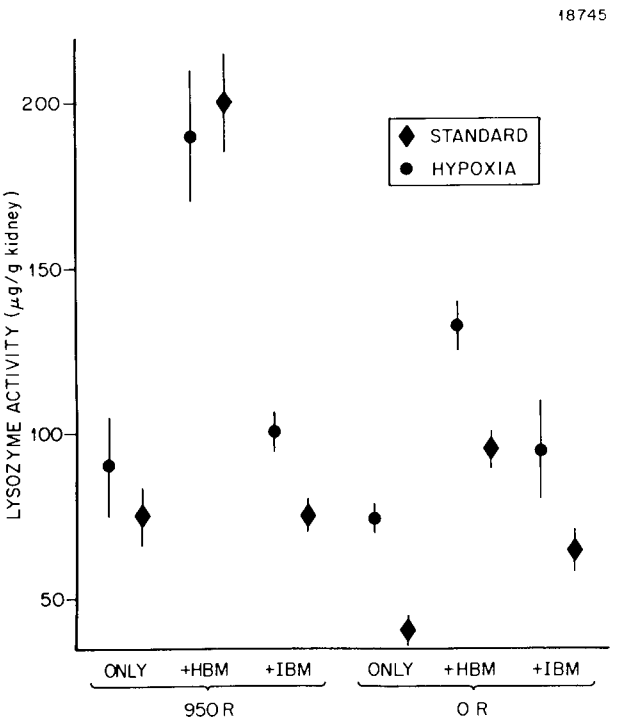

Fig. 9.2.1. Kidney Lysozyme Activity on Day 9. Lines through points show the standard deviation.

not reflect the absence of immunologic activity. The results suggest that additional work is needed on the mechanism of kidney lysozyme elevation during an immune response. The higher lysozyme levels present in all but one hypoxic group, and the lowest level in unirradiated, untreated standard animals suggest that hypoxia, immune response, and experimental manipulation increase kidney lysozyme content.

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9.3 MORPHOLOGIC BASIS FOR LIVER ENLARGEMENT

W. H. McArthur

Enlargement of the liver is one of the early manifestations of bone marrow treatment of lethally irradiated animals. Previous work in this laboratory by Kretchmar *et al.*[1] shows that the peak increase in liver weight occurs on the 12th day following irradiation and bone marrow treatment. If liver cells are enlarged, then nuclei might also be enlarged and show changes in the amount of DNA. Uyeki,[2] however, found no significant increase in DNA of liver nuclei from xenogenic radiation chimeras. The increase in liver weight may be related to enlargement of parenchymal cells or it may be due to increase in number of cells. Such activity of these cells might be indicative of the synthesizing ability of the liver. The purpose of this investigation is to attempt to determine precisely the morphologic basis of liver enlargement.

Twenty male mice were used in these experiments. Each animal was weighed at the beginning of the experiment. They were selected for body weight (between 25 and 32 g) and divided into four groups. The first group was given 940 r x irradiation only; the second group received no irradiation or treatment; the third group was given 950 r total-body x irradiation and within an hour received 40×10^6 nucleated isologous $(101 \times C3H)F_1$ Cum bone marrow cells; the final group was irradiated in the same manner but was given 40×10^6 nucleated homologous (57BL/6F × C3H/Ans ♂) Sim bone marrow cells. At day 12, when peak liver enlargement occurs, all animals were weighed and sacrificed. A specific area of the left lobe was removed, fixed in Zenker's, stained with H & E, and mounted on glass slides. The areas selected for cell and nuclear count are similar from section to section and are randomly chosen. An ocular micrometer was used to measure the size of liver cells and their nuclei.

Table 9.3.1. Cell and Nuclear Size of Liver Cells of Mice

Group	Average Cell Size (μ)	Average Nuclear Size (μ)	Number of Measurements
Normal	21.3	8.7	142 (3 animals)
Isologous	23.0	9.2	148 (3 animals)
X ray	21.7	7.8	43 (1 animal)
Homologous	21.3	8.4	256 (5 animals)

No differences in cell and nuclear size of liver cells among the four groups were observed (Table 9.3.1). The numbers of binucleate cells in normal, x-irradiated, IBM-, and HBM-treated animals were compared. Experimental animals (IBM and HBM) had binucleate cell counts similar to those of normal animals. X-irradiated and IBM animals had twice as many binucleate cells as the normal and HBM-treated animals.

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9.4 KARYO-INTERKARYOMETRY IN LIVER OF RADIATION CHIMERAS

W. H. McArthur

Hepatomegaly of the liver is an early manifestation of bone marrow injection into lethally irradiated mice. It has been suggested by Congdon *et al.*[1] that the enlarged liver may be due to individually enlarged parenchymal cells. Work in progress in this laboratory suggests an increase in the number of all functional cells rather than an increase in the size of individual parenchymal cells.

Craciun[2] suggested a method of karyo-interkaryometry which involves quantitative measurements and cell population dynamics. We have measured distances between nuclei of liver cells from normal and experimental animals.

Four groups of five animals each were studied: normal, x-irradiated, x-irradiated plus isologous bone marrow (IBM), and x-irradiated plus homologous bone marrow (HBM). Each experimental animal received 950 r and 40×10^6 bone marrow cells intravenously. Animals were sacrificed on day 12; and a specific portion of the left lobe of the liver was removed, placed in Zenker's, and stained with hematoxylin and eosin so that a quantitative study and interkaryometric measurements could be made. Distances between nuclei of adjacent cells were measured.

Interkaryometric measurements of adjacent nuclei of liver cells from normal, x-irradiated, and IBM-treated x-irradiated animals were similar and consistent. The range in microns for normal animals was 17.1 to 22.4; for IBM-treated animals 23.2 to 23.9; and for x-irradiated animals 18.7 to 22.2. Measurements of distances between nuclei of two cells separated by a single cell or its process were as follows: normal animals 35.0 to 53.8; IBM-treated animals 44.0 to 47.1; x-irradiated animals 46.4 to 53.8.

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9.5 EARLY PROLIFERATION OF TRANSPLANTED SPLEEN COLONY-FORMING CELLS

A. L. Kretchmar[1] W. R. Conover[1]

Conflicting results have been reported regarding the early proliferation in the spleen of transplanted hemic cells capable of forming gross nodules. When a small number of bone marrow cells were injected into irradiated mice and their spleens subsequently removed and used to prepare cell suspensions which were then injected into other irradiated mice used as recipients in the usual spleen colony assay system, no increase in colony-forming cells could be demonstrated for five days.[2,3] If there is no increase in the number of colony-forming cells during the first four days after transplanta-

tion, then it is difficult to see how erythropoietin could act directly on these cells and produce large erythroid colonies by day 8. A separate primitive hemic cell, sensitive to erythropoietin but not capable of forming spleen colonies,[4] would then seem likely. When larger numbers of bone marrow cells were injected into the primary recipients, however, the number of colony-forming units was found to decrease at first and then increase rapidly with exponential increase after day 3.[5,6] Finally, when a radiobiological technique was used, an increase in survival ratio was found, suggesting that proliferation of colony-forming cells began after a lag of only 24 hr.[5]

Since the number of bone marrow cells injected into the primary recipients could be of critical importance in the experiments summarized above, we assayed the spleens of primary recipients given three different doses of isogeneic bone marrow cells. The experiments also permitted us to determine whether there was a linear relationship between the number of marrow cells injected and the number of colony-forming cells subsequently found in suspensions of the spleens of the primary recipients.

Male and female C3H/UT mice 12 to 16 weeks of age were used. Primary recipients were given 875 r total-body irradiation, and, within 1 to 4 hr, 0.5, 1.0, or 2.0×10^6 bone marrow cells of the same sex and strain were injected intravenously. Six animals from each cell-dose group were killed at various times (Fig. 9.5.1) after treatment and their spleens used to prepare 12 ml of cell suspension. One milliliter of suspension was then injected into each of ten mice irradiated (875 r) 1 to 4 hr before preparation of the cell suspensions. These animals were killed nine days later, and the gross nodules in their spleens were counted. Data from several experiments were combined and are summarized in the figures where the number of secondary recipients used is indicated in parentheses adjacent to each point.

The data from 214 spleens of irradiated mice that had been injected with one-half spleen equivalent from primary recipients given 0.5×10^6 isogeneic bone marrow cells are summarized in Table 9.5.1, where a comparison with expected values assuming a Poisson model is shown. The data deviate significantly from the Poisson model perhaps because the number of colony-forming cells injected is also a random variable.

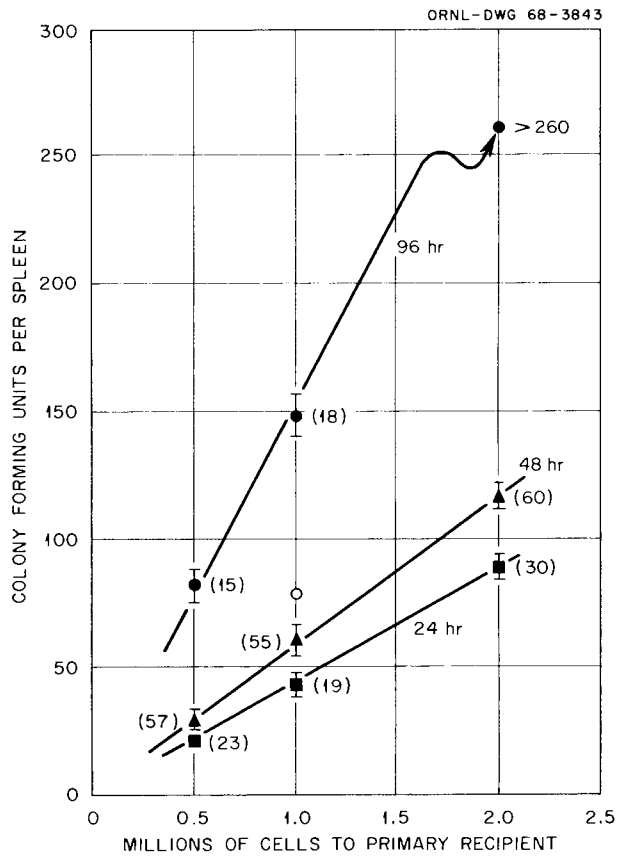

Fig. 9.5.1. Colony-Forming Units in Spleens of Secondary Recipients Plotted Against the Number of Isogeneic Bone Marrow Cells Injected into Primary Recipients. Points are means computed from the number of spleens indicated in parentheses. Vertical lines are one standard error. The number of colony-forming units at 72 hr after 1.0×10^6 cells is shown by O. The point at 96 hr, shown as >260, included spleens with confluent colonies.

Figure 9.5.1 shows that the average number of colonies found in the spleens of the secondary recipients is a linear function of the number of bone marrow cells injected into the primary recipients. Since the slope increases after 24 hr, it is clear that colony-forming cells are proliferating in the spleen after 24 hr. The figure also shows that the increase in colony-forming cells between 24 and 48 hr cannot be detected if fewer than 1.0×10^6 bone marrow cells are given to the primary recipients. These data also show that the doubling time is not constant between 24 and 96

Table 9.5.1. Relative Frequency of Spleens with Various Numbers of Colonies in Irradiated Mice Given One-Half Spleen Equivalent from Mice Given 0.5×10^6 Isogeneic Bone Marrow Cells

Number of Colonies per Spleen, x	Observed Frequency[a]	Expected Frequency[b]
0	0.140	0.086
1	0.206	0.211
2	0.224	0.258
3	0.164	0.211
4	0.149	0.129
5	0.051	0.063
6	0.033	0.026
7	0.023	0.009
8	0.005	0.0028
9	0	0.00075
10	0.005	0.00018

[a]Number of spleens with x colonies divided by 214, which was total number of spleens. Average number of colonies was 2.45.

[b]Calculated from $\frac{2.45^x e^{-2.45}}{x!}$.

hr, being about 48 hr at first and then shortening to 24 hr.

In another experiment all primary recipients were given 1.0×10^6 isogeneic bone marrow cells. Their spleen cells were obtained at 1 or 4 hr and one, two, three, or four days and were injected (one-half spleen equivalent per mouse) into secondary recipients. The number of colony-forming cells decreased during the first 24 hr and then increased by day 4.

The results show that growth in the population of colony-forming cells occurs after 24 hr and that the doubling time in this population is not constant. They also show that the increase cannot be detected if only a few (less than 5×10^5) cells are given to the primary recipients.

The apparent decrease during the first 24 hr may be due to circulation of colony-forming cells with final settling of cells in tissues other than the spleen. If this occurs, the growth rate of the population during the first three days could have been grossly underestimated by experiments of this kind.

The data are also consistent with the suggestion that colony-forming cells in cycle are sensitive to the trauma required to prepare cell suspensions. Since the doubling time of the population is shortening during the first three days, it is possible that the generation time of colony-forming cells transplanted into normal irradiated mice is, at first, relatively long and then becomes rather short. If this is so, then the fraction of the transplanted colony-forming cells that are in a sensitive phase of cell cycle would change during the experiment. Thus, the procedure used might grossly underestimate the number of colony-forming cells. Therefore, while an increase in number of colony-forming cells can be demonstrated during the first three days by transplantation techniques, it is probable that they grossly underestimate the size of the population of colony-forming cells.

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9.6 SHORT-TERM BONE MARROW CULTURES

L. H. Smith T. W. McKinley, Jr.

Previous efforts have been directed toward modification of radiation damage to mouse bone marrow cells in vitro.[1,2] One difficulty encountered in these studies is adequate maintenance of reproductive capacity of normal cells in suspension culture at 37°C – after 6 and 24 hr at this temperature, 25 and 80%, respectively, of the original capacity has been lost. Two modifications of the standard culture procedure[3] were tested. One involved culturing the marrow as a plug rather than as a cell suspension, thereby retaining more

physiological cell-cell relationships. Plugs (~3.0 mg) were placed on Millipore filters, weighed, immersed in culture medium, incubated, and tested for reproductive capacity by the ^{59}Fe spleen method.[4] Quantitative testing is possible because the number of nucleated cells per milligram of bone marrow is quite constant ($\sim 2.5 \times 10^6$). Reproductive capacity of cells in plugs incubated for 6 hr at 37°C, however, was lower (50%) than cells maintained in suspension culture. Since loss of capacity may be related to impairment of diffusion of substances into and out of the plug, a constant-medium flow device is presently being tested. The second modification is one in which a well-oxygenated concentrated isologous erythrocyte preparation (packed cell volume of ~70% in serum) is substituted for tissue culture medium. Six-hour maintenance at 37°C is significantly improved (90%) by use of erythrocytes. Improved survival may be the result of oxygen availability to marrow cells and proximity of marrow cells to the erythrocytes.

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9.7 SIZE OF THE HEMOPOIETIC COMPARTMENT AS A FACTOR IN RADIOSENSITIVITY OF THE MOUSE

L. H. Smith T. W. McKinley, Jr.

Assuming that mortality from whole-body irradiation of mammals over the LD_1 to LD/100 30-day range is a consequence principally of damage to the hematopoietic system, it is attractive to postulate that survival in this exposure range is in part a function of the number of stem cells present at the time of irradiation. A direct test of this postulate requires a means of estimating the number of bone marrow stem cells in each individual prior to irradiation. Presently, however, there is not a reliable method for determining an average stem-cell content, let alone that for each individual. A gross verification of the postulate can be achieved, however, by increasing the number of marrow stem cells (by intravenous injection) before irradiation. Recovery of the marrow-injected mice can be compared with that of irradiation controls. It was found that injection of large numbers of syngeneic marrow cells (100×10^6) about 15 min prior to exposure of mice increased 30-day survival appreciably (Fig. 9.7.1). Similarly, recovery of the erythropoietic compartment in mice given marrow before irradiation with 450 r was more pronounced than in x-ray controls. While it is clear that marrow given before midlethal irradiation enhances recovery and survival, it is not certain that an increased stem-cell pool size is the only factor involved. A tiding-over mechanism may be implicated, in that the injected marrow suspension contains many nonstem cells, for example, young and mature granulocytes, which may enhance recovery of the irradiated animal.

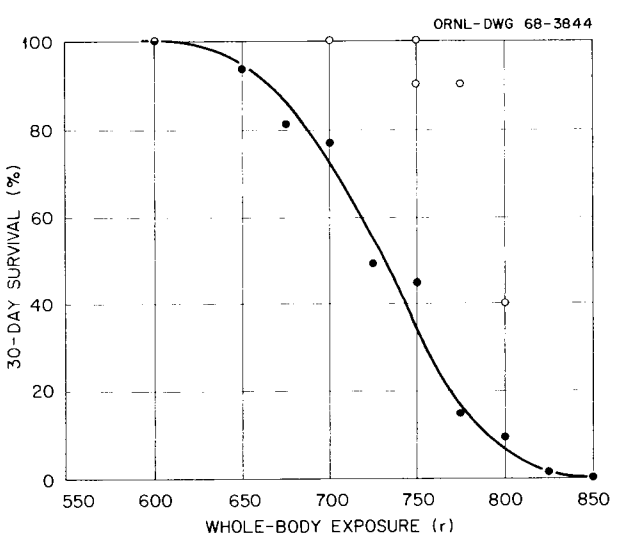

Fig. 9.7.1. Effect of Injecting Bone Marrow Cells Before Irradiation on 30-Day Survival. ● = x-ray only, 20 to 25 mice per point; O = 100×10^6 cells before x irradiation, 10 to 13 mice per point.

9.8 INFLUENCE OF THYMUS CELLS ON ERYTHROPOIESIS OF PARENTAL MARROW IN IRRADIATED HYBRID MICE

Joan Wright Goodman Sarah G. Shinpock

Although the role of the thymus in immunobiology has been intensively and fruitfully investigated in the last ten years, relatively little is known about the influence of this organ on erythropoiesis. That the thymus is in some way involved in control of red cell formation is suggested by (1) the anemia subsequent to neonatal thymectomy of mice, (2) maturation arrest of erythroid cells in neonatally thymectomized opossum, and (3) the association of erythroid aplasia with an epithelial cell tumor of the thymus in man. However, no evidence of anemia is found in mice thymectomized or given multiple thymic grafts as adults, whether or not the animals were stressed by repeated bleedings.

While studying the poor-growth phenomenon in irradiated F_1 hybrid mice that had been given parental bone marrow, we found that certain regimens of pre- or posttreatment with parental cells greatly improved erythropoiesis.[1] Of various organs used as parental cell donors for pretreatment of F_1 hybrid recipients, only those having a high proportion of immunocompetent cells (spleen and lymph nodes) augmented growth of subsequently transplanted parental marrow. Bone marrow, thymus, and liver were ineffective at the cell doses used (up to 3×10^7). However, when given as posttreatment to irradiated hybrids, parental bone marrow cells, exposed to 10,000 r in vitro, were the most effective cell type studied, spleen being second. Dead cells from lymph nodes and thymus produced only marginal improvement of poor growth, whereas liver and red cells had no effect. This report presents experiments in which viable thymus cells, injected into heavily irradiated hybrid mice treated with parental bone marrow, definitely augmented erythropoiesis.

F_1 hybrid mice were given a single whole-body x-ray exposure to 900 r; 18 to 22 hr later they received parental bone marrow (10^6 cells) intravenously, either alone or in combination with thymus cells. A week later ^{59}Fe citrate was administered intravenously, and the uptake of isotope into erythrocytes and spleens was assessed at 24 hr.

Viable cells from parental thymus produced a significant increase in erythropoiesis as shown in Tables 9.8.1 and 9.8.2. Experiments A and B illustrate a small but significant improvement resulting from F_1 hybrid (isogenic with the recipient) thymus cells. When thymus donors were of the second parental strain (C3 in experiment B), uptake values were indistinguishable from those of radiation control mice. High mortality in this group (8/11) suggests that a graft vs graft (GVG) reaction of immunocompetent elements among the many (10^8) thymus cells resulted in rejection of the B marrow transplant. Such GVG activity would obscure or obliterate any beneficial effect that transplanted thymus cells might have on erythropoiesis. The small effect provided by living F_1 cells might represent a "sparing" of marrow cells or a diversion from immunological commitment to red-cell formation. We have no experimental evidence that this was the case and believe it possible that the hybrid cells functioned in a completely nonimmunologic way. Whatever their mode of action, hybrid cells were less effective than parental cells. It is an interesting possibility that parental thymus cells are more effective because they are stimulated immunologically, in graft vs host (GVH) activity, to proliferate to a greater extent than are hybrid cells which recognize no foreign antigens in the host or donated marrow. It is unfortunate that GVG reactions make a detailed investigation of this point by use of second parental or homologous cells an impossibility.

When thymus cells were heavily irradiated, increased erythropoiesis, as judged from ^{59}Fe uptake in recipients, was not regularly seen. As Table 9.8.2 indicates, spleen ^{59}Fe uptake values were significantly but not greatly higher in recipients of dead parental thymus cells than in mice receiving bone marrow only. In one case, experiment F, the red-cell value was also significantly elevated. This implies that whatever it is that thymus furnishes the system to augment erythropoiesis does not require cell replication. However, the low degree of augmentation relative to that produced by living thymus cells shows clearly that ability to proliferate is advantageous in this regard.

Recent studies by Claman *et al.*,[2] reporting a synergistic immunological effect of thymus with bone marrow cells in irradiated mice, may be analogous to our findings. It will be of interest to determine whether thymus cells must be present in hemopoietic tissues to produce these results or whether the effect is humoral.

Table 9.8.1. Effect of Viable Thymus Cells on Erythropoiesis in Irradiated $BC3F_1$ Hybrid Mice Given 10^6 Parental (B) Marrow Cells

Experiment	Donor of 10^8 Thymus Cells	Number of Mice	^{59}Fe Uptake (% of Injected Dose), Mean ± S.E.	
			RBC	Spleen
A	B	31	7.2 ± 0.5	5.1 ± 0.2
	$BC3F_1$	28	2.5 ± 0.3	2.3 ± 0.2
	No thymus	30	1.4 ± 0.1	1.6 ± 0.2
	Radiation control[a]	15	0.3 ± 0.04	0.3 ± 0.02
B	B	22	5.9 ± 0.7	3.1 ± 0.2
	C3	3	0.2 ± 0.1	0.3 ± 0.02
	$BC3F_1$	23	2.5 ± 0.4	1.7 ± 0.2
	$BC3F_1$ (+ no marrow)	5	0.2 ± 0.1	0.2 ± 0.1
	No thymus	28	0.9 ± 0.1	0.5 ± 0.1
	Radiation control[a]	28	0.1 ± 0.01	0.3 ± 0.02
C	B	19	12.3 ± 0.7	5.6 ± 0.2
	B(+ no marrow)	3	1.1 ± 0.4	0.02 ± 0.00
	No thymus	22	3.5 ± 0.4	2.2 ± 0.2
	Radiation control[a]	19	1.2 ± 0.3	0.03 ± 0.00

[a]Received no marrow, no thymus cells.

Table 9.8.2. Comparison of Living[a] with Dead[b] Thymus Cells on Erythropoiesis in Irradiated Hybrids Given 10^6 Parental Marrow Cells

Experiment	Recipient	Donor of 10^8 Thymus Cells	Number of Mice	^{59}Fe Uptake (% of Injected Dose), Mean ± S.E.	
				RBC	Spleen
D	$B6D2F_1$ ♀♀	B6, dead[b]	30	1.5 ± 0.2	1.2 ± 0.2
		No thymus	23	1.2 ± 0.2	0.7 ± 0.1
		Radiation control[c]	14	0.6 ± 0.1	0.3 ± 0.02
E	$B6D2F_1$ ♀♀	B6, living[a]	26	8.2 ± 0.7	4.9 ± 0.2
		B6, dead[b]	29	1.6 ± 0.1	1.6 ± 0.2
		No thymus	29	1.2 ± 0.2	0.6 ± 0.1
		Radiation control[c]	31	0.2 ± 0.03	0.4 ± 0.04
F	$BC3F_1$ ♂♂	B, living[a]	19	6.6 ± 0.7	4.4 ± 0.3
		B, dead[b]	21	3.5 ± 0.5	3.5 ± 0.7
		No thymus	25	1.6 ± 0.2	1.3 ± 0.2
		Radiation control[c]	10	0.1 ± 0.01	0.2 ± 0.02

[a]Eosin-excluding.

[b]Exposed to 10,000 r in vitro.

[c]Received no bone marrow, no thymus cells.

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9.9 MODIFICATION OF GROWTH OF PARENTAL MARROW IN POSTTREATED F_1 HYBRID MICE

Joan Wright Goodman H. Brownell Wheeler[1]

In clinical attempts to achieve long-term survival of transplanted tissues, primary concern has been devoted to the immunologic response of the host. This emphasis on homograft rejection has perhaps obscured the fact that "take" of some transplants may also depend on *non*immunologic parameters, including the functional demand for the graft and other physiologic factors which are as yet undefined. An example of nonimmunologic factors influencing graft survival is the failure of parental bone marrow grafts to grow well in some strains of lethally irradiated F_1 hybrids. These grafts do poorly, even though rejection by the host is impossible and a physiologic need exists for marrow function. We believe that the initial survival of certain transplanted tissues, such as bone marrow, depends on a number of genetically determined factors. These factors may include not only the well-known histocompatability antigens, but also properties of the cell surface, humoral factors required for cell growth, and probably other physiologic requirements. In order to learn more about the nonimmunologic factors which govern initial "take" of bone marrow transplants, we have studied the growth of parental marrow cells in irradiated F_1 hybrid mice under a variety of experimental conditions.

The basic experiment consists in transplanting a small dose of parental bone marrow (10^6 cells) into heavily irradiated F_1 hybrid mice. Assessment of success of the marrow transplant is based on erythropoiesis after one week, as measured by 24-hr ^{59}Fe uptake by erythrocytes and spleens.

Poor growth of parental marrow can be modified by pretreatment of the hybrid with spleen slice grafts or with other preparations of viable, immunologically competent parental cells, isogenic with the subsequent marrow graft, as previously reported.[2] Hybrids so pretreated showed growth of transplanted parental marrow that was equivalent to growth in isogenic recipients. Further investigation of these findings showed that time was required for full development of the pretreatment effect, two to three weeks being optimal, and that the effect was long lasting (at least three months).[3]

In trying to understand why pretreatment with isogenic lymphoid cells should augment marrow growth, we considered the possibility that the pretreatment cells might engage in a graft-vs-host (GVH) response. The effect on subsequent marrow growth could then be either a direct immunologic consequence of the GVH response or an indirect effect resulting from the proliferation of donor cells. If the latter were true and merely the presence of large numbers of parental cells at the time of irradiation and marrow transplantation improved marrow growth, then augmentation of marrow growth should also be observed in other F_1 recipients containing large numbers of parental cells, but not undergoing an active GVH response. This hypothesis was tested by combining large numbers of dead parental cells with the small inoculum of viable parental bone marrow given to lethally irradiated hybrids.

One hundred million dead (exposed to 10,000 r in vitro) isogenic parental cells were added to the living parental bone marrow inoculum (10^6 cells). In these experiments there was no pretreatment prior to irradiation. The results are shown in Table 9.9.1. Parental spleen cells made incapable of division, but morphologically intact, produced a highly significant augmentation of growth of 10^6 living parental marrow cells when given intravenously either 3 hr before or mixed together with the viable inoculum. A small, preliminary experiment, not included in the table, indicated that the dead cells were ineffective if given intraperitoneally. The augmentation was produced specifically by isogenic parental cells. Any nonspecific effect, such as reticuloendothelial stimulation, appears to be ruled out by the fact that isogenic F_1 hybrid and allogeneic spleen cells are completely ineffective. The experiments shown in Table 9.9.2 indicate that dead (10,000 r) parental marrow cells may have a greater growth-stimulating capacity than dead spleen cells, and that as few as 4×10^7 dead marrow cells can potentiate growth of a living marrow graft.

Table 9.9.1. Modification of Poor Growth of 10^6 B6 Bone Marrow Cells in Irradiated[a] $B6D2F_1$ Males by Addition of Dead B6 Spleen to the Marrow Inoculum

Dead B6 Spleen Cells[b]	Viable[c] B6 Bone Marrow	Number of Mice	^{59}Fe Uptake (% of Injected Dose), Mean ± S.E.	
			Erythrocytes	Spleen
None	10^6	29	1.2 ± 0.1	0.6 ± 0.08
10^8 (given intravenously 3 hr before bone marrow)	10^6	24	6.1 ± 0.7	5.0 ± 0.6
10^8 (mixed and incubated for 1 hr at 6°C with bone marrow)	10^6	30	6.6 ± 0.6	4.7 ± 0.4
10^8	None	28	0.1 ± 0.03	0.4 ± 0.05
None	None	29	0.1 ± 0.01	0.4 ± 0.04

[a]Recipients exposed to 900 r one day before transplantation.
[b]Cells exposed to 10,000 r in vitro.
[c]Cells excluding eosin.

Table 9.9.2. Modification of Poor Growth of B6 Marrow Cells by Dead[a] Cells in Irradiated[b] $B6C3F_1$ Mice

Dead B6 Cells[a]	Viable B6 Marrow Cells	Number of Mice	^{59}Fe Uptake (% of Injected Dose), Mean ± S.E.	
			Erythrocytes	Spleen
Male Recipients				
None	10^6	25	3.7 ± 0.3	2.2 ± 0.3
Bone marrow, 10^8	10^6	20	18.3 ± 1.3	9.8 ± 0.5
Spleen, 10^8	10^6	21	10.6 ± 0.9	7.3 ± 0.6
None	None	20	0.4 ± 0.1	0.3 ± 0.02
Bone marrow, 10^8	None	17	0.5 ± 0.2	0.3 ± 0.05
Spleen, 10^8	None	23	0.4 ± 0.1	0.3 ± 0.02
Female Recipients				
None		30	1.5 ± 0.1	1.1 ± 0.1
Bone marrow, 2×10^7	10^6	31	2.0 ± 0.2	1.7 ± 0.2
Bone marrow, 4×10^7	10^6	25	2.9 ± 0.4	2.6 ± 0.3
Bone marrow, 8×10^7	10^6	21	5.5 ± 0.7	5.0 ± 0.5
None	None	15	0.1 ± 0.02	0.5 ± 0.05
Bone marrow, 8×10^7	None	7	0.1 ± 0.02	0.4 ± 0.05

[a]B6 cells exposed to 10,000 r in vitro, mixed with viable cell inoculum, given intravenously.
[b]Recipients exposed to 900 r whole-body dose.

In the experiments reported above, any procedure which provided a large number of morphologically intact parental cells in the F_1 host at the time of irradiation and marrow transplantation markedly potentiated the "take" of a parental marrow graft. Provision of an equivalent number of cells from an unrelated strain had no effect on marrow growth. Two possible mechanisms come to mind in considering these findings: (1) marrow cells can grow only when the host contains large numbers of parental cells which provide compatible isogenic cell surfaces, and are possibly inhibited in their growth by direct contact with foreign cell surfaces; (2) a nutritional or humoral requirement exists for growth of parental marrow cells which is not available in the F_1 hybrid but can be furnished by the isogenic parental cells present in the irradiated hybrid host (in a feeder-layer manner). Our data do not allow us to distinguish between these or other possible mechanisms at the present time. Further investigation along these lines should clarify the nature and relative importance of the nonimmunologic factors which govern the growth of transplanted cells.

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[1]Department of Surgery, Harvard Medical School; and Veterans Administration Hospital, West Roxbury, Mass.

[2]Joan Wright Goodman and H. B. Wheeler, *Nature* **211**, 1420–21 (1966).

[3]Joan Wright Goodman and H. B. Wheeler, *Transplantation* **6**(2), 173–86 (1968).

9.10 A METHOD OF CLOSED GAS ANESTHESIA FOR LABORATORY ANIMALS

M. L. Simmons L. H. Smith

A method was developed for anesthetizing mice and rats and maintaining them in a level of surgical anesthesia for relatively long periods of time up to several hours. The model 960 veterinary anesthetic machine[1] was used, and the anesthetic chosen was Metofane.[2]

In ordinary anesthesia, animals are individually intubated with tracheal tubes and fastened directly to the machine. The closed system of anesthesia is so called because all gases exhaled by the animal (oxygen, anesthetics, and carbon dioxide) are retained within the system and are not vented to the surrounding atmosphere. The exhaled gases are recirculated to the animal by means of check valves that permit only unidirectional flow. During recirculation the gases are passed through soda lime, where the carbon dioxide produced by the animal is absorbed. Because it is a closed system, sufficient oxygen must be admitted to meet the requirements of the animal.

This system was modified to accommodate small laboratory animals. A closed-chamber adapter system attachment was constructed as shown in Fig. 9.10.1. Because the breathing volume of inhaled and exhaled gases could not be used for gas exchange as it is with a larger single animal, we attached the chamber as an enlargement in the circle. The system is diagrammed in Fig. 9.10.2. Small laboratory animals, specifically mice and rats, can be placed inside the chamber and the depth of their anesthesia controlled by limiting the amount of anesthetic vaporized into the circular flow pattern.

The anesthetic chosen was methoxyflurane, a halogenated ethyl methyl ether (2,2-dichloro-1,1-difluoroethyl methyl ether). It contains 0.01% butylated hydroxytoluene as an antioxidant. Methoxyflurane is nonexplosive, is low in toxicity, and exhibits a wide margin of safety after a level of surgical anesthesia is reached.

This system has been very successful and has many potential uses. Specifically, we have used it as an anesthetic for splenectomy of the mouse. Over 300 splenectomies have been performed with only two deaths.

References

[1]Pittman-Moore, Indianapolis, Ind.

[2]Pittman-Moore trade name for methoxyflurane.

9.11 ANESTHESIA IN LABORATORY RATS

M. L. Simmons J. B. Jones

A new product called Innovar,[1] which is a combination of Sublemaze (a narcotic analgesic with a pharmacologic spectrum similar to morphine) and Inospine (which is a nonnarcotic tranquilizer), has been used in this laboratory in rats.

Fig. 9.10.1. Closed System Adaptation of Veterinary Anesthesia Machine.

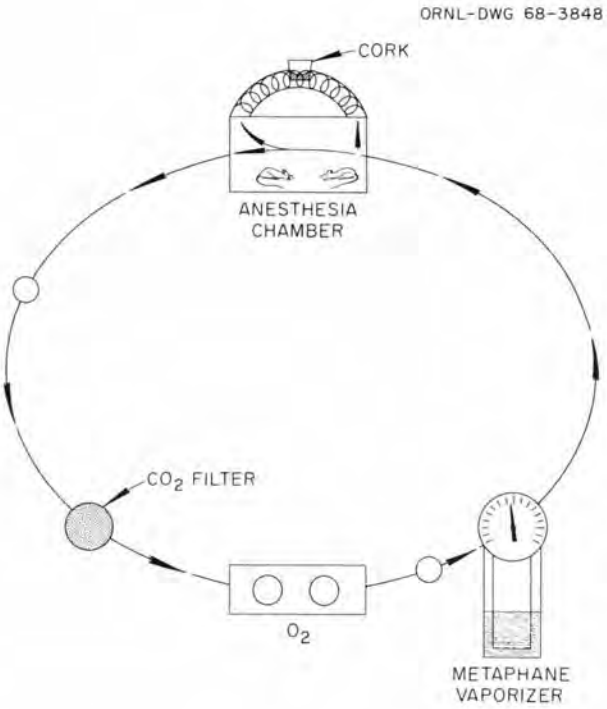

Fig. 9.10.2. Diagram of Closed System Adaptation of Veterinary Anesthesia Machine.

Thirty-one rats, 15 Sprague-Dawley and 16 Long-Evans, weighing from 30 to 480 g and varying in age from 24 days to 7+ months were used. Induction time averaged 8.7 min. The animals ceased moving about and rather than relaxing, as is usually expected with anesthesia, became somewhat rigid. They usually remained in the position one placed them. Analgesia was profound, but animals responded to loud noises at all dose levels. Five of eight animals receiving 0.01 cc per 100 g required an additional dose before surgery could be performed without evidence of pain. All animals receiving 0.02 cc or more, per 100 g, remained taped to the surgery board with absolutely no movement or evidence of pain. A muscular twitch occurred in a few animals at the incision site. One Long-Evans rat, 7+ months old, weighing 380 g, died several hours after splenectomy (Table 9.11.1). No young adult animals showed adverse effects at either 0.01 or 0.02 cc per 100 g of body weight. The dose of 0.02 cc per 100 g was optimal.

Eleven animals were given the same dose range, but not subjected to splenectomy. This group showed the same responses as those undergoing surgery, though one animal died 7 min after re-

Table 9.11.1. Innovar Anesthesia in Rats

Derivation	Dose Rate per 100 g (cc)	Number of Animals Used	Splenectomy	Survival
Long-Evans	0.01	6	3/6	6/6
Long-Evans	0.02	7	5/7	6/7[a]
Sprague-Dawley	0.02	6	6/6	6/6
Long-Evans	0.03	3	2/3	2/3
Sprague-Dawley	0.03	9	4/9	9/9
		31	20/31	29/31

[a]Death occurred in splenectomized animal.

ceiving Innovar. This group included three animals weighing 20, 25, and 30 g. They appeared insensitive to pain at the same dose as heavier, older rats though more movement was noted.

The manufacturer recommends the product only for dogs, and it is a common drug used for anesthesia in cesarian sections in that species. The drug is well tolerated by the rats, and they remain sedated for approximately 45 min. We have premedicated with atropine to prevent salivation and bradycardia. However, it does not seem to be necessary, as animals not premedicated with atropine responded equally well. The drug can be used in combination with ether, methoxyflurane, sodium pentobarbital, and other general anesthetics.

Because the drug can be given intramuscularly and has a relatively quick effect, it is ideal for use with rats requiring surgical or other manipulation. Interestingly enough, when we tried this drug with mice it only caused excitement and eventually death. The manufacturer states that undesirable effects have also been noted in cattle, sheep, cats, and horses.

Reference

[1]McNeil Laboratories, Fort Washington, Pa.

9.12 REPLICATION OF A MINUTE DNA ANIMAL VIRUS

R. W. Tennant K. R. Layman R. E. Hand, Jr.

Kilham rat virus (KRV) is a member of a small group of icosahedral animal viruses which range only 180 to 220 A in diameter. Although it was isolated originally from tumor-bearing rats, the virus has not been shown to have oncogenic characteristics. However, KRV in vivo appears to replicate preferentially in tissues with actively proliferating cells. Further, infection of rats or hamsters in utero or shortly after birth gives rise to facial bone deformities, hepatitis, and cerebellar ataxia.

Evidence indicates that the viral DNA is a double-stranded molecule with a molecular weight of about 2×10^6 daltons. This represents a genome probably capable of coding only three to five proteins. These characteristics prompted this inquiry into the replication of KRV.

Strain 171 of KRV was obtained from Dr. Lawrence Kilham, and a stock pool was prepared in Sprague-Dawley rat embryo cell cultures. The virus was assayed by hemagglutination of guinea pig RBC or by plaque assay in culture of Fisher strain rat embryo cells. Generally, stationary monolayer cultures ($\sim 4 \times 10^6$ cells) are infected with KRV (2×10^8 pfu/ml) and incubated at 37°C for 2 hr.

The replication cycle of KRV in primary Sprague-Dawley strain rat embryo cells, infected at a multiplicity of about 10, is shown in Fig. 9.12.1. Cell-associated infectious progeny virus appeared between 5 and 10 hr post infection and reached a plateau by about 22 hr. This increase is paralleled by the accumulation of hemagglutinins intracellularly and an increase in hemagglutinins in the supernatant fluid, which indicates that progeny virus probably is released soon after assembly.

Fluorescent antibody staining of cells after infection with KRV revealed that the efficiency of infection was approximately 20% and that the en-

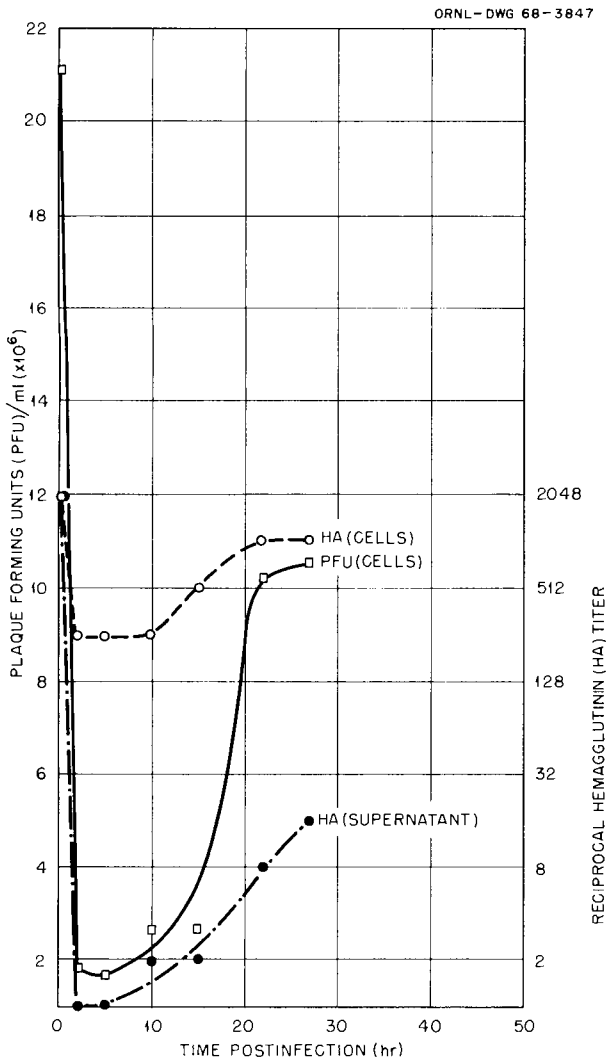

Fig. 9.12.1. Replication of KRV in Sprague-Dawley Rat Embryo Cells.

tire process of replication probably occurs in the nucleus. Some granular cytoplasmic fluorescence seen late in the replication cycle may represent movement of completed virions.

Although the Sprague-Dawley strain rat embryo cells support virus replication, which leads to extensive cytopathic effects, it was not possible to achieve conditions which allowed the expression of viral plaques. The rats themselves are free of antibody to KRV and respond to immunization with high-titer antiserum. Recently we have found that plaques develop readily in cells obtained commercially, which are derived from Fisher-strain rats. The reason for this difference is not apparent and will be examined in subsequent experiments.

Experiments were performed to determine the patterns of macromolecular synthesis in virus-infected Sprague-Dawley rat embryo cells. First, we measured the incorporation of ^{3}H-thymidine (TdR) into trichloroacetic acid (TCA) insoluble material at various periods after infection. Replicate cultures were exposed to the labeled precursor for 1 hr and the cells were washed, sonified, and washed in cold 5% TCA on filter disks. The pattern of incorporation, shown in Fig. 9.12.2, revealed the following: Control cultures, although they have reached a stationary phase of growth, incorporate ^{3}H-TdR after mock infection (with virus-free vehicle) and medium change, in a cycle which reaches a peak at 20 to 25 hr. Infected cells demonstrate, however, a decrease in rate of incorporation as early as 2 hr post infection. While a slight increase in incorporation is

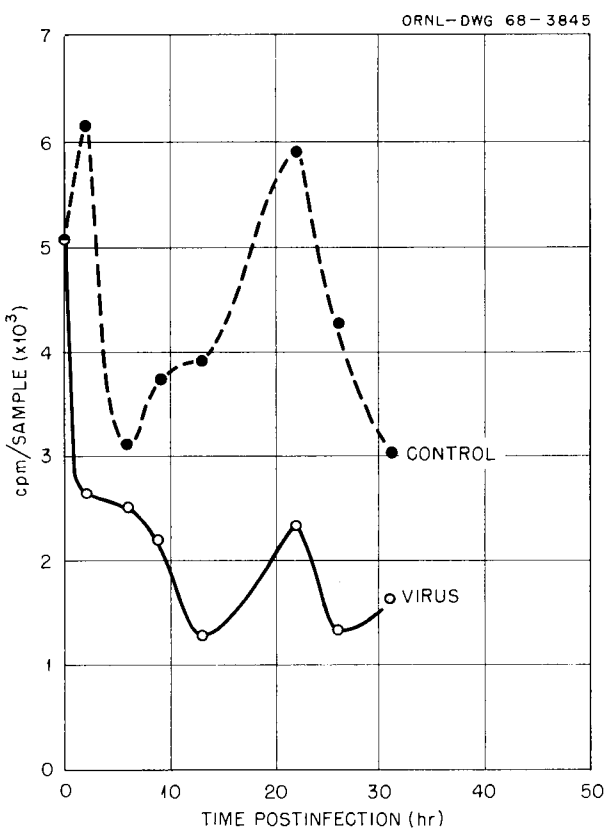

Fig. 9.12.2. Effect of KRV on ^{3}H-TdR Incorporation in Rat Embryo Cells.

seen to correspond to that of control cells, it never exceeds half the rate of controls.

Neutralization of virus by specific immune serum, however, abolishes the effect of the virus on ^{3}H-TdR incorporation. The rate of incorporation of ^{3}H-uridine is also affected within 2 hr after infection, but less than that of ^{3}H-TdR, and incorporation of ^{14}C-leucine remains unaffected in both infected and control cultures throughout the infectious cycle.

The rapid effect of virus on ^{3}H-TdR incorporation and its inhibition by virus neutralized with immune serum suggests adsorption of the virus itself might alter the ability of cells to incorporate ^{3}H-TdR. However, such a difference should be apparent in the pool sizes, and no such difference was seen. It is possible also that the viral nucleic acid competes more favorably for the cell polymerase or that a viral-coded protein inhibits an essential cell function. These possibilities are currently being studied.

Kilham rat virus undergoes a relatively rapid replication cycle in rat embryo cells, the entire process apparently completed in the cell nucleus. The virus has a rapid effect on incorporation of thymidine and to a less extent uridine, but does not affect incorporation of leucine into cell macromolecules.

9.13 INCIDENCE OF MURINE VIRUS ANTIBODY IN HUMANS IN CONTACT WITH LABORATORY ANIMALS

R. W. Tennant Roberta K. Reynolds
K. R. Layman

Several viruses indigenous to mice and rats have been shown to be widely disseminated in experimental and breeder colonies,[1] and there is evidence that some of these viruses may be capable of replication both in mice and men. For example, reovirus antibody is found frequently in human sera, and virus isolations have been made although no syndrome has been defined. The potential cross infection by some other common murine viruses was examined in this study by testing the incidence of antibody in the serum of individuals who have had various degrees of contact, ranging from two months to over 15 years, with conventional animals compared with those of a noncontact sample.

The viruses and serological techniques used in this study have been described elsewhere.[2] Briefly, sera were tested for antibody against mouse encephalomyelitis (Theiler's GDVII), reovirus type 3, pneumonia virus of mice (PVM), polyoma virus, newborn pneumonitis (K virus), Sendai, Toolan H-1 (hamster osteolytic virus), and Kilham rat virus by the hemagglutination-inhibition test. Antibody against mouse hepatitis and mouse adenovirus was assayed by the complement fixation (CF) test. All viruses and specific antisera were monotypic reagents purchased from Microbiological Associates, Inc. Human sera were obtained from 92 donors from the Biology Division and from 112 individuals not normally in contact with animals. All sera were coded prior to testing. Sera were adsorbed with guinea pig, mouse, and sheep cells for the appropriate tests and treated with neuraminidase prior to testing against Kilham rat virus and Toolan H-1 virus. All tests were performed with the *Microtiter* system and were rejected when positive and negative control sera or antigen and complement titrations varied more than twofold in dilution or more than two CH_{50} complement units.

In neither group was antibody found against PVM, polyoma, K virus, Kilham rat virus, Toolan H-1 virus, mouse adenovirus, or mouse hepatitis viruses. However, antibody to reovirus type 3, Sendai, and GDVII virus was detected in the sera of all individuals tested, regardless of group. Analysis of the frequency distribution of antibody titers against these viruses in the two groups revealed general coincidence, and in no case were the differences statistically significant according to Student's *t* test (Fig. 9.13.1).

As has been reported previously,[3,4] antibodies against reovirus 3 and Sendai were detected. The former may represent response to actual infection, while the latter probably is an antibody response to parainfluenza virus type 1, which is immunologically related to Sendai virus. While all sera tested reacted with these antigens, the significant feature is that the distribution of titers between the groups was so similar that it was not indicative of increased risk of infection by contact with experimental animals. No additional risk could be detected, however, if individuals possessed antibody prior to contact with the animals.

Another finding of interest is the occurrence of antibody to GDVII virus in both test groups. It is important to determine the specificity of this hemagglutination inhibitor, since this group of

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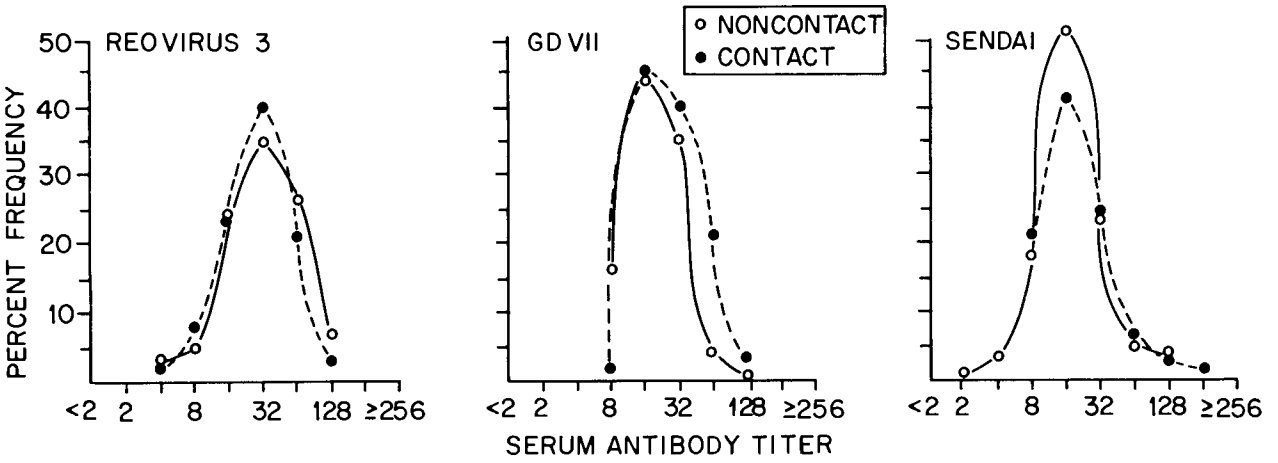

Fig. 9.13.1. Percent Frequency of Serum Antibody Titers in Contact and Noncontact Groups.

murine viruses is not known to cross-react immunologically with known human viruses. It may represent actual human infection with the encephalomyelitis viruses or with an unidentified virus which shares immunological characteristics with GDVII.

The absence of detectable antibody against the seven viruses listed above probably means that the capsid proteins are not replicated in humans. However, in the case of the mouse hepatitis antigens, the sensitivity of our test system may be marginal, since Hartley *et al.*[5] have reported that antibody was found in human sera against certain hepatitis strains.

In summary, these results provide no evidence of transmission of virus from laboratory rodents to humans. Immunological evidence for infection of humans by murine encephalomyelitis viruses or agents sharing immunological reactivity has been found, subject to future tests for specificity.

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9.14 OBSERVATIONS ON NORMOBARIC HYPOXIA ENCEPHALOPATHY IN MICE

C. B. Richter R. E. Toya, Sr.

Most studies of brain damage related to oxygen deficiency have employed dysbaric conditions, or temporary or permanent interruption of part of the blood supply to the brain. Observations on a normobaric hypoxia have been made in such instances as anesthetic accidents, severe secondary anemias, and CO_2 poisoning in man. It has been observed that mice kept in hypoxia chambers under normobaric conditions showed clinical signs of central nervous system damage.[1] Sudden ischemia selectively necroses cerebral and cerebellar cortex areas, while protracted hypoxemia causes necrosis of the basal ganglia and brain stem structures.[2] The question of the sequence of events preceding the observed clinical or pathological manifestations is not entirely clear. Ischemic or hypoxemic induced vasospasm with subsequent thrombosis is a frequently proffered hypothesis in the case of hypoxia or anoxia. However, ultrastructural studies have disclosed, in the case of anoxic ischemia at least, that irreparable damage occurred in nerve cells before irreparable damage occurred in endothelial cells in the brain.[3]

Previous experiments with the hypoxia chamber[4] showed a greater susceptibility of the female over the male to clinical evidence of central nervous system damage.

Thirty-nine female mice of the hybrid strain ($B6C3F_1$), 12 weeks of age, were used in this experiment. The animals were housed in chambers equipped with dimethyl silicone rubber membrane to obtain hypoxia.[5] For this experiment, eight mice were housed in each chamber, giving an oxygen concentration of 5 to 6%. They were kept under hypoxic conditions for 14 days and then removed and maintained under routine laboratory conditions. Food and water were freely available through the course of the experiment. Six days after being removed from the hypoxia chamber, daily serial sacrifice was begun. The heads of the sacrificed mice were fixed in neutral buffered formalin. Blood was obtained for hematocrits and hemoglobin determination at the start of the experiment, seven days after the start of the experiment, and at the time of sacrifice.

Oxygen content in the chamber was measured daily by a Beckman oxygen analyzer. Hematocrits and hemoglobin were determined by standard methods.

Mice were categorized according to clinical signs in the following manner: (1) animals that had not shown clinical signs, (2) animals that showed early clinical signs, (3) animals that had shown the clinical signs and were allowed to remain in the experiment.

The mice were quite depressed in the hypoxic environment, and activity and food and water consumption were greatly reduced. Figure 9.14.1 gives the variation in hemoglobin content and hematocrit over the course of the experiment. The first clinical evidence of disease was seen on day 18, with the peak attack rate occurring on experiment day 24 (26%), ten days after returning to a normal environment. Clinical evidence of disease consisted of symptomatic torticollis, uncontrolled rotational movements (rolling), ataxia, or twisting when picked up by the tail. There was one fatality in this experiment. Previous hypoxia chamber experiments experienced minor mortality also. Beginning on day 20, mice were selected for sacrifice, and every day thereafter two to four mice were destroyed until day 31, when the last survivors were killed.

Pathological Findings. – Studies here are incomplete as yet; however, pathological changes were found in mice belonging to all three of the sacrifice groups. Lesions were located in the basal areas and brain stem, and none was seen in the cerebellar or cerebral cortex. Lesions consist of focal necrosis with diffuse areas of glial cell necrosis in the surrounding neuropile. Neuronal necrosis, demyelination, and axonal degeneration occurred in varying degrees in the damaged areas. Also outstanding in damaged tissue were degeneration of blood vessel walls, stasis, and hemorrhage. Most interesting in damaged vessels and capillaries is the accumulation of granular material on the inner surface of the vessel wall (Fig. 9.14.2). If further studies point to a nervous tissue origin for this material, as is expected, it is quite likely that it plays an important roll in initiating the sequence of events leading through vessel damage to stasis and hemorrhage.

Discussion. – Since pathological changes would likely precede clinical onset by some time, it is interesting that mice examined at day 14 from a previous experiment had only scattered glial cell necrosis under light microscopy. This implies that the hypoxic insult initiates irreparable changes that progress even after return to the normal environment, or that return to the normal environment itself is the initiating factor to real damage. The latter seems unlikely in view of the observations of others.[2] The pathogenesis of the disease must be more fully clarified, as well as sites of predilection and topographical distribution. Since pathological changes were not seen in visceral organs, the well-known first-order susceptibility of brain tissue to hypoxia is merely reinforced.

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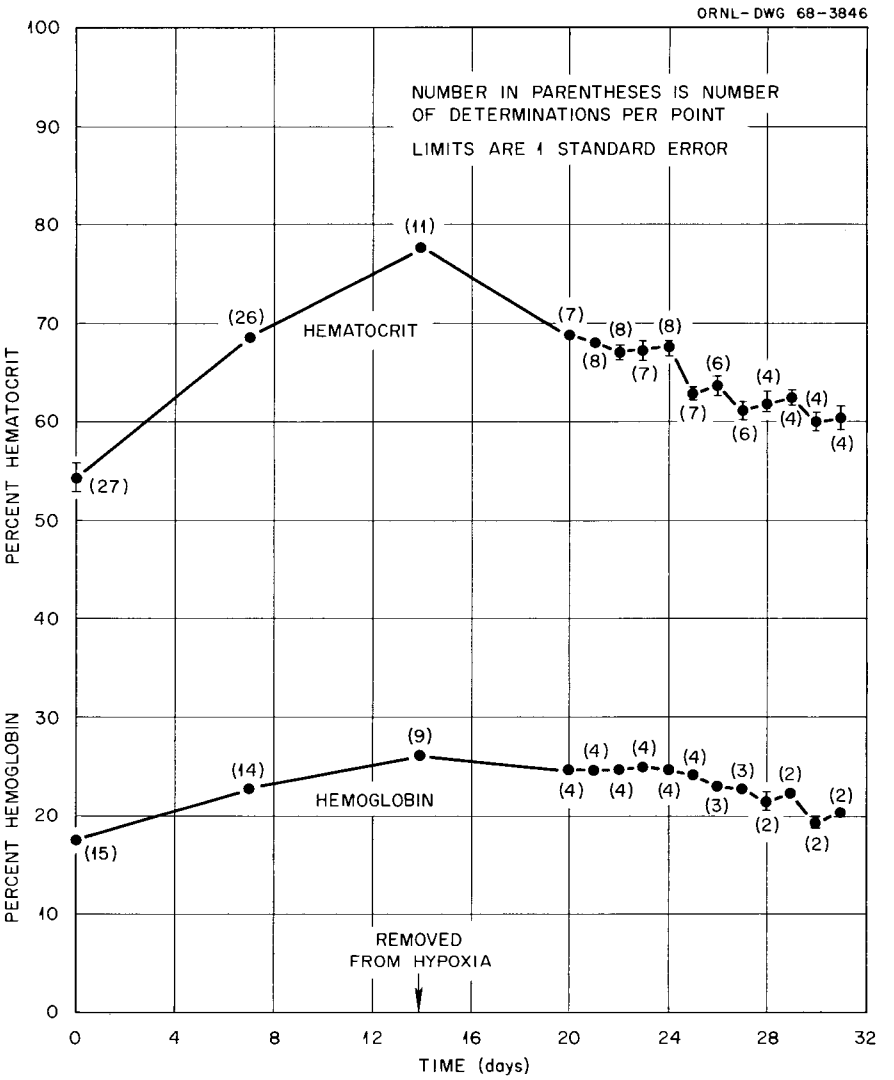

Fig. 9.14.1. Hematocrit and Hemoglobin Values for Hypoxic Mice.

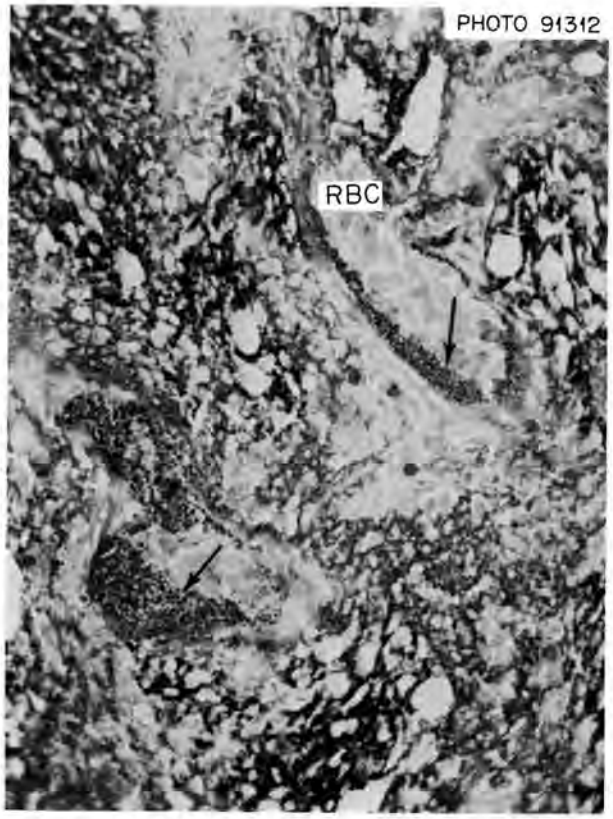

Fig. 9.14.2. Caudate Nucleus Mouse Without Clinical Signs. Arrows indicate granular material on inner wall of blood vessels. Gomoris aldehyde fuchsin. 400×.

9.15 SOME NORMAL BLOOD VALUES FOR MICE

W. J. Miller C. B. Richter

A study was made on 16 strains of mice received from Cumberland View Farms to determine some of their normal blood values. Values determined were: total white cell and red cell counts, hemoglobin, total serum protein, serum albumin, and mean corpuscular hemoglobin (Table 9.15.1). Ten male mice with an average age of 60 days were used from each strain. The purpose of the study was to provide base-line information for investigators, and compare results with the published information.

Mice were anesthetized with Metofane, and the thoracic cavity opened. Two syringes (1 cc tuberculin) were partially filled by direct cardiac puncture. The first syringe was unheparinized, and the collected blood was allowed to clot for serum protein determination. The second syringe was heparinized, and the collected blood sample was used for the hemoglobin determination and total white and red cell counts. The hemoglobin value was determined by the cyanomethemoglobin method.[1] The white and red cell counts were made on a Fisher Autocytometer. All dilutions were made with lambda pipets, and colorimetric determinations were made on a Beckman model 150 spectrocolorimeter using ultramicrotechniques. The serum from the clotted sample was further separated by centrifugation. Part of this serum was used to determine the total serum protein by the biuret method,[1] and the remainder was washed with ether to precipitate albumin. Albumin values were also determined by the biuret method. The mean corpuscular hemoglobin was calculated by using the derived red blood cell count and hemoglobin values. All measurements were made on individual mice.

One interesting note was the marked difference in hemoconcentration in blood collected from the heart and blood collected from the tail or orbital plexus of the same mouse (not published). The total red cell values in blood collected from the tail by the method outlined by Russell or from the orbital plexus approximated her results.[2] Blood collected by direct cardiac puncture had total red cell values in the order of 2 to 4 million less.

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Table 9.15.1. Some Normal Blood Values for Cumberland View Farms Mice

Strain	WBC[a]		RBC[a]		Hb[b] (g/100 ml)		Serum Protein[c] (g/100 ml)		Albumin[c] (g/100 ml)		MCH ($\mu\mu$g)	
	Mean	Range	Mean	S.E.	Mean	S. E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
			$\times 10^6$	$\times 10^6$								
C3H/Anf Cum	3235	2300–4,500	6.24	0.23	12.99	0.18	5.98	0.12	4.96	0.20	21.35	0.36
C57BL/Cum	4710	3400–6,600	6.30	0.16	13.31	0.21	6.07	0.09	3.59	0.15	21.21	0.44
CBA/Cum	3725	2300–5,400	6.97	0.31	13.74	0.21	6.34	0.18	4.20	0.12	20.30	0.97
BALB/c Cum	4010	3150–7,300	7.74	0.25	15.60	0.33	6.55	0.13	3.88	0.21	20.23	0.68
C3H/An Cum	4855	3200–8,150	5.35	0.37	13.56	0.38	7.15	0.13	Not done		23.54	1.32
C57BL/6 Cum	5940	3900–9,800	5.19	0.50	12.63	0.25	5.62	0.04	3.86	0.16	22.90	2.10
DBA/2 Cum	3283	2500–3,900	7.15	0.23	13.90	0.32	5.53	0.03	3.87	0.15	19.47	0.37
101/Cum	4964	2800–9,800	7.97	0.31	14.50	0.61	6.52	0.10	4.77	0.07	18.77	0.62
CAF_1/Cum	2938	2150–5,400	5.95	0.17	13.84	0.32	5.29	0.08	3.17	0.14	23.44	0.77
AKR/Cum	6335	3600–9,800	5.53	0.28	12.19	0.60	6.41	0.17	4.07	0.19	22.23	0.59
C3BF1/Cum	6200	5300–9,200	3.86	0.18	11.44	0.15	5.93	0.10	3.77	0.15	30.24	1.43
C31F1/Cum	5362	3850–11,800	4.79	0.24	13.71	0.26	5.79	0.10	4.43	0.12	29.04	1.21
B6D2F1/Cum	4487	3100–9,200	5.81	0.21	12.72	0.26	6.34	0.11	3.95	0.04	22.11	0.67
ICR	4061	2500–7,000	6.05	0.13	14.10	0.21	6.21	0.12	3.35	0.19	23.32	0.73
BC3F1/Cum	3012	2600–3,800	5.87	0.22	13.06	0.34	5.41	0.11	4.07	0.12	22.41	0.76
CD2F1/Cum	3345	2450–4,500	8.62	0.14	13.89	0.25	5.65	0.10	3.86	0.13	16.54	0.39

[a]Counts made on Fisher Autocytometer employing heparinized heart blood. All dilutions were made by using lambda pipets.

[b]Cyanomethemoglobin method.

[c]Biuret method.

10. Radiation Immunology

Takashi Makinodan

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10.1 RIBONUCLEIC ACID SYNTHESIS OF PHYTOHEMAGGLUTININ-TRANSFORMED LYMPHOCYTES IN VITRO

Hiroo Yamamoto Takashi Makinodan
R. A. Brown

The sequential pattern of nucleic acid synthesis by peripheral blood lymphocytes, induced by phytohemagglutinin (PHA) stimulation, has been studied as a model for lymphocyte transformation in an immune response. Optimum conditions for separation of the peripheral lymphocytes, their culture, and the stimulation by PHA have been established.

The transformation of PHA-stimulated human peripheral blood lymphocytes to blast cells, as indicated by the rate of DNA synthesis, is preceded by a parallel increase in RNA synthesis. RNA and DNA synthesis were maximal at 72 hr. When the RNA of the lymphocytes was labeled with ^{14}C-uridine and extracted in the presence of added unlabeled HeLa cells, sucrose density gradient analysis showed RNA with sedimentation values of approximately 28, 16, and 4s. At 20 hr label was associated with the 4 to 10s

fraction of the cold carrier HeLa cell RNA. At 72 hr all classes were labeled. The results would suggest that an important early event in the PHA stimulation of lymphocytes is the induction of the synthesis of new messenger RNA.

10.2 TIME-DEPENDENT CYTOLOGICAL STUDIES OF ANTIBODY-FORMING CELLS EXPOSED TO 10,000 r OF X RAYS

Toshihiko Sado

Previously[1,2] it has been shown that exposure of spleen cells in Millipore diffusion chambers to 10,000 r during the plateau phase of secondary antibody response resulted in a decrease in the total number of cells and a relative increase in the proportion of plasma cells without any significant change in the rate of antibody synthesis. This would suggest that the antibody-forming cells were spared, and if so, this irradiated culture system should be very useful for biochemical and ultrastructural studies of mechanics of antibody synthesis and secretion at the subcellular level. In order to characterize this irradiated culture system further, we addressed ourselves to the following questions: (1) What fraction of the total specific antibody-producing cells can survive for several days after 10,000 r? (2) What is the nature of DNA-synthesizing cells observed several days after irradiation? Can they proliferate and generate more immunocompetent progeny? (3) What kind of nuclear and cytoplasmic changes can be detected ultrastructurally in antibody-forming cells several days after irradiation?

The general procedure was similar to that described previously.[1] Briefly, diffusion chambers containing 24×10^6 dispersed primed mouse spleen cells and 24×10^5 sheep RBC were implanted into the peritoneal cavities of 600-r-irradiated genetically compatible mice. Six days later chamber-bearing mice were exposed to 10,000 r of x rays, and then the chambers were transferred into the second set of 600-r-treated mice. Sham-irradiated control chambers containing identical aliquots of spleen cell plus sheep RBC suspension were run simultaneously. Individual antibody-producing cells were detected by the direct and indirect hemolytic plaque assay methods developed by Jerne *et al.*[3] and Wortis *et al.*[4] For ultrastructure studies of irradiated spleen cells, cells from several chambers were pooled and fixed in either (1) 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 1 to 2 hr, followed by postfixation with 1% buffered osmium tetroxide (pH 7.4) for 1 hr, or (2) 1% buffered osmium tetroxide. After embedding in epon, sections ranging in color from silver to pale gold were prepared from LKB Ultrotome III using glass knives. The sections were then double-stained with saturated uranyl acetate and 0.4% lead citrate (pH 12.0) and were examined with Hitachi electron microscope, model HU-11B, operated at 75 kv.

Table 10.2.1 summarizes the number of direct and indirect plaque-forming cells (PFC) scored four days after the irradiation. It can be seen

Table 10.2.1. Specific Antibody Producers (PFC) Recovered from Chambers Four Days After Exposure to 10,000 r[a]

Mean ± 1 standard error

Group	PFC/Chamber		Total Nucleated Cells Per Chamber	PFC per 10^6 Cells	
	Direct	Indirect		Direct	Indirect
			$\times 10^6$		
Negative control[b]	108 ± 14	22 ± 5	2.28 ± 0.36	59 ± 13	13 ± 4
Positive unirradiated control	23,325 ± 5,806	327,325 ± 73,887	2.68 ± 0.42	7,265 ± 2,029	126,868 ± 29,164
Irradiated with 10,000 r	10,434 ± 2,343	142,290 ± 34,158	0.45 ± 0.08	24,203 ± 4,317	315,485 ± 39,215

[a]X ray given six days after culture.

[b]Negative control: primed spleen cells + isologous mouse RBC.

that exposure of six-day cultures to 10,000 r resulted in only twofold reduction in the number of specific antibody producers per chamber. However, it was found that the frequency of PFC in irradiated cultures (PFC per 10^6 cells) was three times higher than that of the unirradiated controls. In fact, three out of ten nucleated cells were found to be specific antibody producers in these irradiated cultures in contrast to one out of ten in the unirradiated control. Autoradiographic studies revealed that a significant number of immature plasma cells were still capable of incorporating ^{3}H-thymidine as late as eight days after the irradiation. However, these thymidine-incorporating cells did not generate more immunocompetent progenies, as they were shown to degenerate during or after metaphase. Electron microscopic studies showed that in most cases fully developed endoplasmic reticulum associated with numerous ribosomes and Golgi complexes of fully differentiated mature plasma cells remained intact, whereas alteration of nuclear structure was fairly pronounced (Figs. 10.2.1 and 10.2.2). When the cells were fixed with osmic acid alone, irradiated cells harvested four days after irradiation showed ruptured plasma membranes, which were only occasionally seen in the unirradiated cells. Apparently irradiation caused some change in the membrane which was magnified during fixation with osmic acid, because this was not shown clearly in cells fixed with combined glutaraldehyde and osmic acid. Current studies involve incorporation of ^{3}H-labeled amino acids by these antibody-forming cells by utilizing electron microscopic autoradiography.

References

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[3]N. K. Jerne *et al.*, p. 109 in *Cell Bound Antibodies*, ed. by B. Amos and H. Koprowski, Wistar Institute Press, Philadelphia, 1963.

[4]H. H. Wortis *et al.*, *Immunology* **11**, 603 (1967).

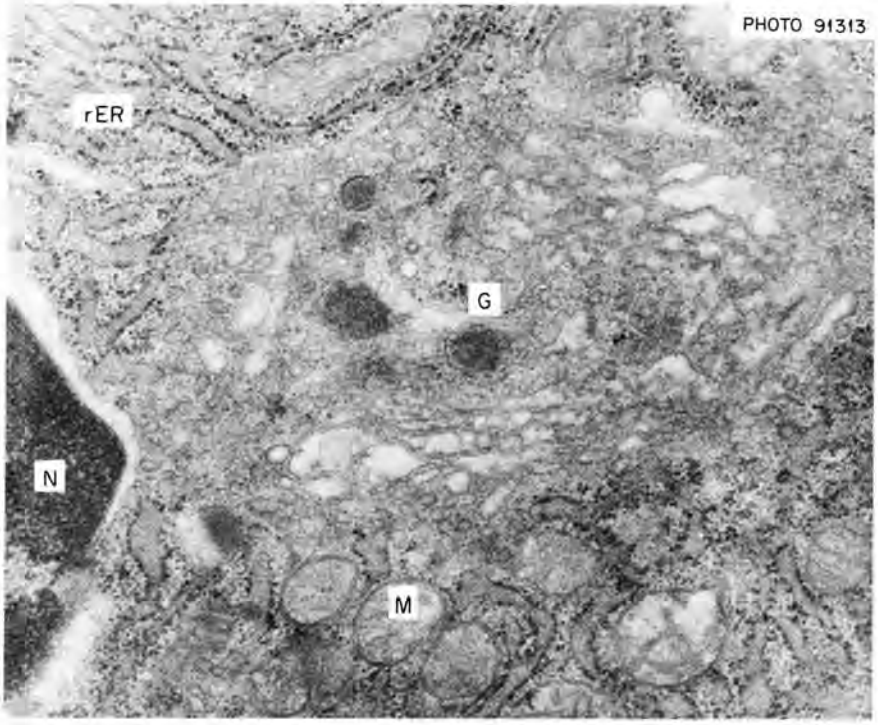

Fig. 10.2.1. A Part of the Golgi Complex (G) of Mature Plasma Cells Four Days After Exposure to 10,000 r. Note also apparently normal mitochondria (M) and endoplasmic reticulum associated with ribosomes (rER). 35,000×.

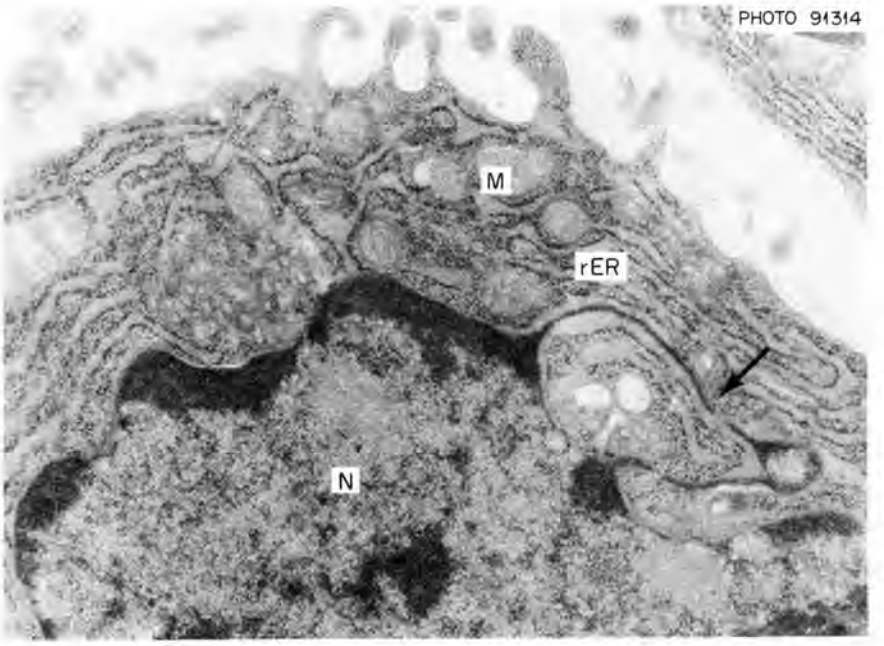

Fig. 10.2.2. A Part of the Nucleus Manifesting Alteration of Nuclear Envelope (Arrow). 24,300×.

10.3 THE ENUMERATION OF ANTIBODY-FORMING CELLS IN LYMPHOID TISSUE FOLLOWING ANTIGEN STIMULATION

Glenda C. Webb E. H. Perkins

The method of detecting individual hemolytic antibody-producing cells directly[1] and indirectly[2] has made it possible to assess more simply antibody production at the cellular level. Therefore, it would seem desirable to assess the relative contribution of the various lymphoid tissues in a given immune response, because information of this nature is necessary to understand regulation of antibody production. With this objective in mind, the number of direct and indirect hemolytic plaque-forming cells (PFC) was assessed for the spleen, lymph nodes (cervical, axillary, brachial, renal, mesenteric, inguinal, lumbar, and caudal), thymus, bone marrow, peritoneal fluid, and blood of mice at various intervals after primary and secondary antigen sheep red blood cell injection.

Excellent time-dependent splenic PFC response profiles were obtained for both direct and indirect PFC following an intravenous injection of 10^9 sheep erythrocytes (Fig. 10.3.1). Only a few direct or indirect PFC were found in other lymphoid tissues. The data clearly show that, although the spleen constitutes only approximately one-third of the total lymphoid tissue mass in mice, it contributes over 99% of the hemolytic antibody-forming cells. Although not all organs and lymphoid tissue were tested (that is, Peyer's patches, deep mediastinal lymph nodes, and lungs were not tested), it would seem that the tissues tested were representative samples of lymphoid tissue, and one would therefore not expect to find numerous plaque-forming cells in other sites. If this reasoning is valid, then one must conclude that the spleen is the major and only significant site of antibody synthesis following intravenous injection of sheep erythrocytes in the mouse.

References

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[2]D. W. Dresser and H. H. Wortis, *Nature* **208**, 859 (1965).

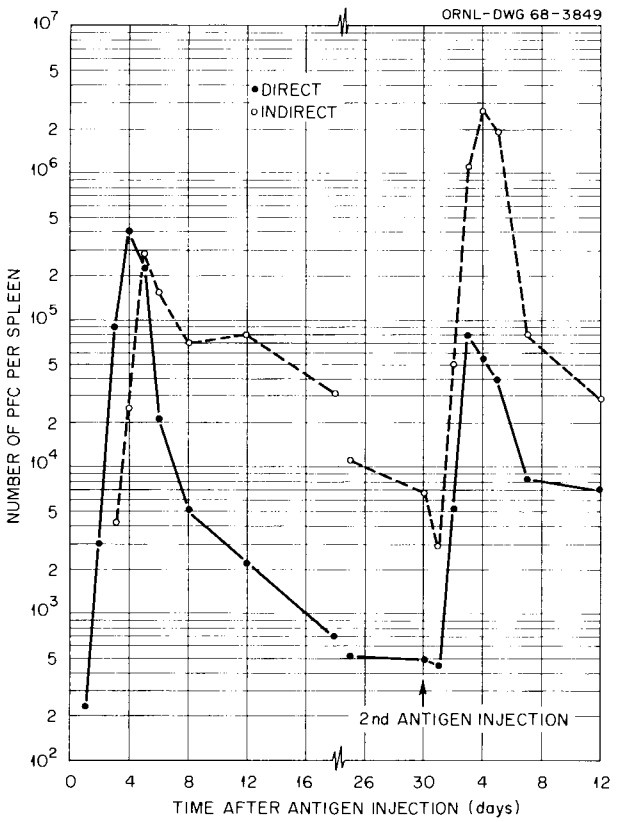

Fig. 10.3.1. Primary and Secondary Response Profile of Hemolytic Antibody-Producing Cells of the Spleen.

10.4 GROWTH AND DIFFERENTIATION OF MURINE MYELOMA CELLS IN DIFFUSION CHAMBER CULTURE

Dona C. Hooper J. F. Albright

In studying those cytokinetic parameters involved in the growth and differentiation of neoplastic tissues, it has been difficult to distinguish which changes in the tumor cell population are due to interaction with host cells and which are the property of the tumor cells themselves. We wish to study the properties of a tumor cell population separated from interactions with host cells, yet having free exchange with the circulation. To do this, we have cultured transplantable murine myelomas in diffusion chambers (porosity 0.22 μ) implanted in host mice. We have compared myeloma tumors producing Bence-Jones and γG globulins.

Studies of tumor cells recovered at various times after initiation of culture have yielded the following information:

1. Chamber cultures of cells derived from solid tumors secreting Bence-Jones or γG globulins were compared. In each case, the cell number recovered showed an initial decline, followed by a rapid rise to a level higher than the inoculum size, and a subsequent plateau. The initial rate of cell loss was characteristic of the tumor. The doubling time from day 2 to day 4 of culture was 48 hr for both types of cells over the range of 2.5 to 50 $\times$ 10^6 cells/ml. The plateau level was directly related to the size of the initial inoculum.

2. The invasiveness of tumor cells subcultured from chambers was studied. During rapid growth in chambers the cells were most active as measured by the number of cells required to provoke a tumor. Cells from cultures in the plateau phase were distinctly less active in provoking tumors.

3. Circulating factors in immune animals do not substantially alter the course of growth in chambers, although they are known to suppress tumor growth when the cells are inoculated subcutaneously.

4. The proliferative capacity of cells dissociated from solid tumors has been examined by evaluating the proportion of "stem" cells. The minimum number of cells required to give rise to 10^5 or more progeny was determined. For the two different tumors studied, this number was substantially different.

5. The relation between the minimum number of cells which would grow in chambers and the minimum number required to give rise to tumors was also investigated. For tumor MPC 47, as few as five cells can give rise to a colony when isolated in a chamber; 10^5 cells of this tumor are needed to produce a new tumor upon subcutaneous injection.

These experiments show clearly that the host environment complicates the analysis of intrinsic factors that regulate growth of a tumor. If myeloma cells derived from solid tumors are physically restricted and protected from host cell interaction,

there is apparent "control" of their growth. We intend to pursue this line of research in order to assess more clearly this apparent "differentiation" of a tumor in the special condition of diffusion chamber culture. These studies will provide data that should be significant in resolving questions concerning progenitor cells and cytodifferentiation of tumors as well as methods for alteration of inhibition of tumor growth.

10.5 A LOW-MOLECULAR-WEIGHT ANTIBODY

R. A. Brown J. M. Ellis

In the process of developing red blood cell stroma as a sensitive immunoabsorbent for detecting broad classes of antibodies during the early immune response, a low-molecular-weight antibody has been detected in mouse sera. An attempt has been made to characterize this material and to do the necessary control experiments.

The low-molecular-weight antibody is an α_2 globulin with sedimentation coefficient of 4S and a molecular weight of approximately 50,000. It appears to consist of one chain similar to an H chain. It probably does not agglutinate red blood cells. The binding constant for the antigen is small; the antibody can be lost by extensive washing.

4S antibodies against homologous as well as heterologous, but not isologous, stroma are found in normal mice. There is a small increase in the level of the 4S antibody three days after injection of antigen. A large specific increase in the level of the 4S antibody can be obtained by transferring primed spleen cells into an irradiated recipient without antigen. If the recovered 4S antibody is concentrated by lyophilization, a small amount can be reabsorbed specifically.

Although other investigators have detected the presence of low-molecular-weight antibodies[1] in sera, it does not appear likely that we are describing the same phenomenon. The 4S antibody could be a preexisting recognition factor.

Reference

[1]Ralph C. Williams, Jr., and Karl Schmid, *J. Immunol.* **99**, 406 (1967).

10.6 TOXIC FACTORS IN THE SPLEENS OF AGING MICE AND PROLONGATION OF LIFE BY SPLENECTOMY

J. F. Albright Julia W. Deitchman
Takashi Makinodan

In aging mice the steady decline in immunological capacity results primarily from depletion of antigen-stimulable progenitor cells capable of producing antibody-synthesizing progeny.[1] This fact, coupled with the observation that the mean life-span is considerably less when mice are reared in an environment rich in infectious organisms, implicates the failure of the immune system as a contributor to senescence and death. Therefore, it seems reasonable that immunologic replenishment might significantly prolong life. Preliminary straightforward attempts have been only moderately successful owing to several complicating problems. The major one of these is that the spleens of aging animals become progressively imbued with toxic factors, as will be illustrated in this report.

Mice (BC_3F_1 strain) were reared under controlled conditions until they were ~75 weeks old. At this time they were exposed to 850 r x radiation and injected intravenously with 10^6 bone marrow cells from 14-week-old syngeneic donors. In addition, some groups of mice received varying numbers of dispersed spleen cells from syngeneic donors that were either 14, 112, or 137 weeks old. Shown in Fig. 10.6.1*a* is the fact that the survival time of recipient mice was inversely related to the age of the mice that served as spleen cell donors. As few as 5×10^3 cells taken from 112-week-old donors reduced survival time by 50% as compared with animals that received cells from 14-week-old donors (comparing times of 50% survival). The survival time of mice that received cells from 137-week-old donors was reduced by 92%. These results suggested that splenectomy of aging mice might prolong survival. Figure 10.6.1*b* shows that this is so. The experiment was performed upon mice that were ~100 weeks old. One group was splenectomized; a second group was surgically manipulated but the spleen left in situ; the third group was untreated. Splenectomy resulted in an 18% increase in overall life-span (time of 50% survival). Or, viewed in another way, it nearly doubled the remaining time of survival of the population of mice beyond 100 weeks of age. Surgical insult that did not include

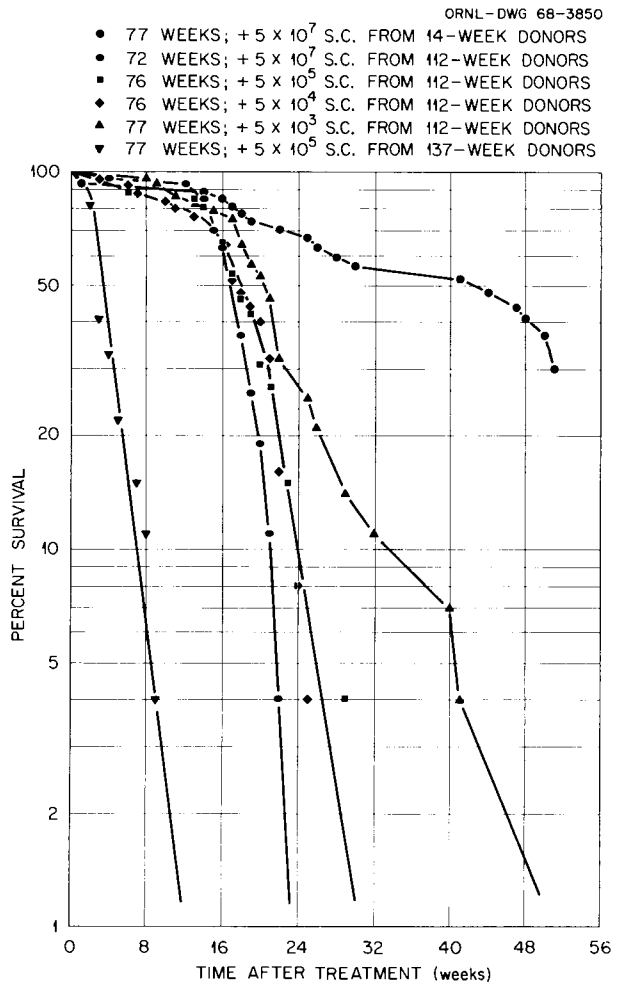

Fig. 10.6.1a. Survival of BC_3F_1 Mice When Treated at 75 Weeks of Age with 850 r X Radiation, 10^6 Bone Marrow Cells from Syngeneic 14-Week-Old Donors, and Spleen Cells from Syngeneic Donors of Different Ages.

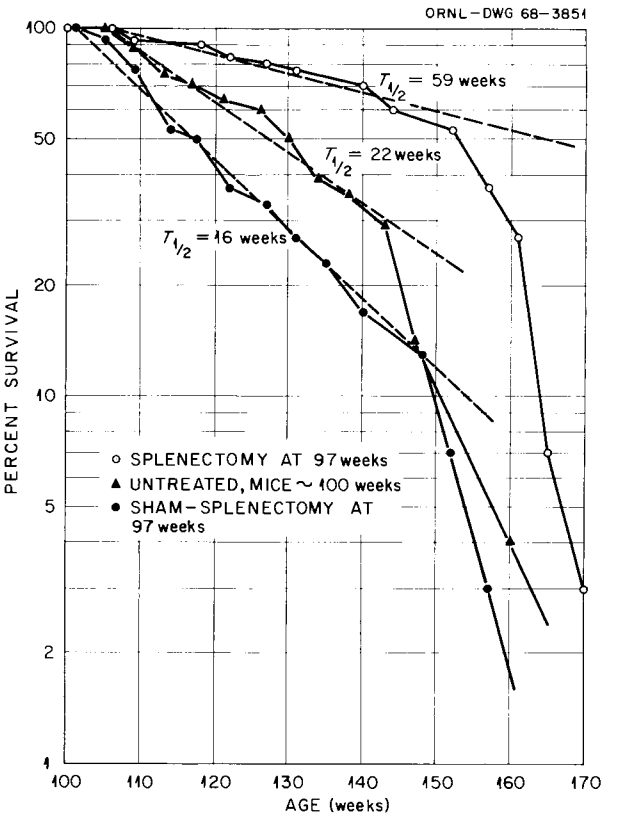

Fig. 10.6.1b. Survival of BC_3F_1 Mice at ~100 Weeks of Age.

removal of the spleen significantly reduced remaining time of survival.

There are good theoretical reasons for believing that repopulation of mice with suitable progenitor cells – particularly those that will effect immunological replenishment – should result in extended life expectancy. However, it is likely that several technical barriers will be faced before such an objective can be reached. The removal of the barrier that has been discussed here appears reasonably easy in that removal of the spleen at an optimum age should remove the principal source of the toxic factors. Further analysis of the factors may result in methods for inhibiting or delaying their appearance.

Reference

[1]T. Makinodan and W. J. Peterson, *J. Immunol.* **93**, 886 (1964).

10.7 ASSESSMENT OF HEMOLYTIC PLAQUE-FORMING CELLS IN MILLIPORE DIFFUSION CHAMBERS

D. L. Groves

Bussard and Anderson[1] have shown that plaque-forming cells (PFC) can be recovered from Millipore diffusion chambers containing mouse spleen cells undergoing a primary immune response. The number of PFC demonstrated, however, was

quite small. This problem of low yield was investigated from two standpoints: the method of recovery of cells from chambers and the possible "toxic" effect of chambers on culturing efficiency. Recovery of PFC from chambers is made difficult by the formation of a clot which retains the majority of the cells. Since others[2] had demonstrated the usefulness of pronase in dissolving the clot, this enzyme was employed in attempting to increase the number of recoverable PFC.

The length of time which PFC could remain in 0.5% pronase at room temperature was first determined because of their sensitivity to this enzyme. It was found that inactivation of PFC commenced in about 7 min. Due to this rapid inactivation of PFC by pronase, means of quenching the enzymatic activity were needed. Dilution of the pronase-treated cell suspension in cold 10% bovine serum albumin was found satisfactory.

Attempts to improve the culturing efficiency of chambers involved pretreatment of chambers by soaking them in 1 or 10% isologous mouse serum or 30% ethanol. Only pretreatment with 10% serum for about 24 hr had a significant effect, resulting in over a threefold increase in the number of PFC recovered as compared with untreated chambers. A marginally significant increase in the total number of nucleated cells recoverable was also found. Suspension of the cell inoculum in 10% serum and using untreated chambers would not supplant pretreatment of the chambers.

By employing pretreated chambers and recovering cells through the use of pronase, it was possible to obtain $\sim 10^3$ PFC per 10^6 nucleated cells recovered at the peak of the response with an input of 10^7 normal spleen cells. The magnitude of this primary response is comparable with that seen in the intact mouse.

With improved culturing efficiency and methods for removing cells from chambers, it is now possible to conduct experiments involving the accurate enumeration of antibody (hemolysin)-producing cells in Millipore diffusion chambers. Studies of a primary immune response initiating from a *single* progenitor cell in a chamber are now plausible in that it should be relatively easy to detect the antibody-synthesizing progeny of these individual cells.

References

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10.8 THE REGULATION OF ANTIBODY PRODUCTION

D. C. Vann

The classical antibody-response profile exhibits a plateau phase in which the amount of circulating antibodies remains nearly constant over a period of time. This level of circulating antibodies may be regulated by homeostatic mechanisms operating on antibody-producing cells at one or more of the following levels: (1) antibody synthesis, (2) secretion of intracellular antibodies, and (3) the antibody-producing cell population. It is possible that this regulation is effected by direct negative feedback from the level of circulating antibodies. This present study has measured the effect of high levels of specific antibodies supplied by host animals on the antibody-synthesizing activity of spleen cells cultured with antigen in Millipore diffusion chambers.

Millipore diffusion chambers initially containing 2×10^7 spleen cells from mice primed with sheep red blood cell (SRBC) and 2×10^6 or 2×10^5 SRBC were recovered from host animals at various times after implantation and incubated in vitro in the presence of ^{14}C-leucine to measure the rate of appearance of newly synthesized antibodies as described previously.[1] To assess the effect of high levels of host antibodies to SRBC on the initiation of the antibody response in these chambers, experimental hosts had received 850 r followed by injection of 10^8 primed spleen cells and 10^9 SRBC seven days earlier. These animals had hemagglutinating antibody titers of approximately 2^{15} at this time. Control animals had high antibody titers to rat RBC or were normal sublethally irradiated mice. It was found that the chambers in control animals mounted a vigorous antibody response three to four days after implantation which continued for more than 14 days. Chambers implanted in animals with preexisting high levels of antibodies to SRBC failed to initiate an antibody response. To determine the effect of increased antibody levels on plateau-phase antibody production, diffusion chamber cultures were initiated in normal animals, and

7 to 13 days after implantation these chambers were transferred to high-antibody hosts as described above. Sham transfer of chambers in normal animals served as controls. When assayed for production of new antibodies one or two days after transfer, experimental chambers synthesized antibodies at the same rate as control chambers, although the host antibodies raised the serologically determined antibody levels in the chambers to approximately 20 times the normal level. The antibodies synthesized in both groups were of the 7S type.

The techniques described here have enabled the direct assessment of the rate of antibody production by spleen cells under conditions in which the level of free antibodies can be varied. By these means it has been possible to demonstrate that high levels of preexisting antibodies inhibit the initiation of a secondary immune response. It has not been possible to demonstrate rapid inhibition of an ongoing antibody response by raising antibody levels 20-fold above their normal levels. Therefore, under these conditions antibody levels do not appear to regulate the synthesis or secretion of new 7S antibodies by fully differentiated antibody-forming cells. It is possible that the action of the exogenous antibodies on the initiation of the immune response was through combining with the SRBC antigenic sites and thereby blocking their immunogenic action.

Reference

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10.9 EVIDENCE FOR SYNTHESIS OF LOW-MOLECULAR-WEIGHT INCOMPLETE ANTIBODY BY SPLEEN CELLS OF AGED MICE

Takashi Makinodan W. J. Peterson
R. A. Brown J. M. Ellis
E. H. Perkins Shirley C. Tipton

Previous serological studies[1] revealed that the antibody-forming capacity of aged mice is ~10% of that of young adult mice. The defect at the cellular level was due primarily to a decrease in the number of antigen-sensitive progenitor cells. The capacity of antibody-forming cells also seemed to be altered to the extent that 7S antibody formation was more severely curtailed than 19S antibody formation, so far as the biologically active complete antibody is concerned. Formation of the biologically less active incomplete antibody was not assessed in these studies. Therefore, there is the possibility that, unlike antibody-forming cells of the young adult, they may be defective additionally, in that they may be synthesizing a significant amount of incomplete antibody. The present study was undertaken to determine the capacity of spleen cells of the aged to synthesize complete and incomplete antibodies.

Primary and secondary antibody-forming capacities of spleen cells of male and female BC_3F_1 mice ranging in age from 27 to 42 months were studied by injecting intravenously 10^9 sheep or rat red blood cells (RBC). At various intervals from 1 to 14 days thereafter, the spleens were removed, weighed, and assessed for antibody formation. This was done by dicing the spleen into 2- to 3-mm pieces and culturing them in vitro for 4 hr in the presence of ^{14}C-leucine. The culture fluid was then dialyzed exhaustively and absorbed with an excess of RBC stroma, which served as our immunoabsorbent. The antibody was then eluted off the stroma at acid pH, neutralized, and centrifuged through a sucrose density gradient, and the various fractions were analyzed for isotopically labeled antibody. When sheep RBC was used as an antigen, $\frac{1}{3}$ to $\frac{1}{2}$ of the spleen was assessed for the presence of direct and indirect plaque (hemolysin-type complete antibody) forming cells according to the method of Jerne[2] and Wortis.[3]

Based on in vitro antibody synthesis, the findings were as follows: (1) Spleen cells of the aged responded to antigen stimulation more slowly than those of the young adult; that is, the maximum activity occurred two weeks after antigen injection in contrast to one week. (2) Most of the antibody synthesized was low-molecular-weight 4S type, which is not synthesized normally at the height of response by spleen cells of the young adult. This type of antibody can be classified as incomplete antibody, because it cannot be detected by the usual serological methods (agglutination, hemolysis, etc.). (3) At the height of response the primary antibody-forming activity was comparable with that of the young adult, and the secondary antibody-forming activity was

$^1/_{16}$th of that of the young adult. Based on the number of hemolytic plaque-forming cells, the kinetics of primary response was comparable with that of the young adult; that is, the maximum response occurred within a week after antigen stimulation. However, the response was only $^1/_{30}$th of that of the young adult. The secondary response differed in that the maximum response occurred ten days after antigen injection, but the response was only $^1/_{70}$th of that of the young adult.

The results show that antigen-stimulated spleen cells of the aged cannot generate sufficient numbers of cells synthesizing hemolysin-type complete antibody. This agrees with our earlier observation based on assessment of agglutinin-type complete antibody formation. However, the most significant finding is that antibody-forming cells of the aged are abnormal in that they synthesize more of the 4S type of incomplete antibody than the young adult. It is possible that 4S antibody may lack the F_c fragment. If so, this would be evidence in support of the theory that the H chain of immunoglobulins is made up of two peptide chains, each under the control of independent genes.

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[1]T. Makinodan and W. J. Peterson, *Proc. Natl. Akad. Sci. U.S.* **48**, 234 (1962); *Develop. Biol.* **14**, 112 (1966).

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10.10 FURTHER CHARACTERIZATION OF A CELL TRANSFER SYSTEM FOR THE STUDY OF ANTIGEN-SENSITIVE PROGENITOR CELLS

M. J. Bosma[1] E. H. Perkins
Takashi Makinodan

One of the better methods to assess the relationship between the relative number of antigen-stimulated progenitor cells and serum antibody output is to inject intravenously a mixture of a known number of dispersed lymphoid cells and an optimum dose of antigen into heavily x-irradiated syngeneic, and therefore immunologically unresponsive, recipients and then determine time-dependent serum antibody titers (cell transfer or in vivo culture method). However, this method of assay is subject to reservation, because infused progenitor cells can clone in different tissues of the host, and it is conceivable that the kinetics of antigen-stimulated cellular changes can vary according to the local microenvironment of the tissue in which the progenitor cells are cloned. Hence, it would seem desirable to assess serum antibody output by cells cloning only in the spleen, which is one of the more favorable colonizing sites when lymphoid cells are transferred intravenously. A series of cell transfer studies was therefore carried out to determine (1) what fraction of the time-dependent serum hemagglutinin content is due to immunocompetent cells cloned in the spleen, (2) what fraction of the total progenitor cells transferred cloned in the spleen, and (3) whether the number of hemolytic plaque-forming cells (PFC) generated per antigen-stimulated progenitor cell is dependent upon the dose of lymphoid cells transferred.

This was done by first assessing the relative response of recipient mice splenectomized and sham splenectomized 1 hr to two days after transfer of dispersed primed and nonprimed spleen cells. This measures the serum response of cells cloned in sites other than the spleen. The percentage of the total serum antibody response by progenitor cells cloned in the spleen and nonsplenic sites was deduced from the altered response profiles. In this way we found that about 90% of the antibodies in the serum during the first week after cell transfer are synthesized by progenitors which cloned in the spleen. This conclusion was substantiated by the results of the following experimental approach. Irradiated recipient mice with exteriorized lead-shielded spleens were given additional irradiation (800 r) 5 to 6 hr after spleen cell transfer. Mice were then bled at various intervals and serum hemagglutinin titers determined. The results showed that the hemagglutinating antibody response profile curve of the spleen-shielded mice was comparable with the sham control group which did not receive additional irradiation.

Having obtained this information, we determined the fraction of injected immunocompetent cells which cloned in the spleen. This was done by the well-known serial spleen cell transfer scheme. The relative number of immunocompetent cells

was assessed both in terms of the antibody synthesized and the number of antibody-synthesizing cells in the spleen. The results showed that about 10% of the injected progenitors cloned in the spleen. We then demonstrated that the number of PFC generated per antigen-stimulated progenitor cell cloned in the spleen was constant only when the number of spleen cells transferred was greater than 12.5×10^6. When fewer spleen cells were transferred, the number of plaque-forming cells generated per antigen-sensitive progenitor cell was sharply reduced.

Reference

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11. General Physiology

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11.1 CONTROL OF CELL DIVISION IN *E. COLI* FILAMENTS

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The bacterium *Escherichia coli* AB 1899 NM is a potentially useful organism for studying the control of cytokinesis. Ultraviolet or ionizing radiation induces the formation of long multinucleate filaments which lyse without cell division or the formation of macroscopic colonies.[2] Cross septation in these filaments can be induced by a variety of chemical and physical agents, including cell extracts;[3] thus a study of the effects of these agents may provide information on the control of cell division in *E. coli*.

We have reported that cell-free extracts of *E. coli* induce cross septation in 1899 NM filaments. The extracts can be fractionated by centrifugation into a heat-labile particulate fraction and a heat-stable dialyzable fraction.[2] The soluble fraction alone did not induce cross septation but was required for maximal activity with the particulate fraction. We have further characterized the particulate fraction. Differential and sucrose gradient centrifugation indicate that the particulate fraction sediments at about 100 S. The active material can be recovered from CsCl density gradients at a density of approximately 1.2. Currently we are analyzing the active materials chemically and by electron microscopy. The sensitivity of the material to enzymatic digestion is also being tested.

A number of chemical compounds have division-promoting activities. The most effective agents are sulfhydryl compounds such as glutathione and mercaptoethanol, which are active at 10^{-4} to 10^{-3} *M*. Other substances such as pantoyl lactone, acetate, and propionate are active but only at much higher concentrations, 10^{-2} to 10^{-1} *M*.

The bacterium *E. coli* 1899 NM has distinct advantages for cell division studies. It is easily assayed by colony counts, since the induction of cell division leads to a macroscopic colony. Further growth is clearly dissociated from cytokinesis. During filament formation the overall growth[2] and macromolecular synthesis proceed at a rate comparable with normally dividing cells. It may be possible, therefore, to study cytokinesis uncomplicated by growth.

The significance of materials in cell extracts for the control of cell division is still unclear. However, the observation that a particulate fraction which can be isolated as a discrete fraction on sucrose density gradients justifies further ef-

forts to characterize and identify the fraction and its possible role in cytokinesis.

The unique effectiveness of very low concentrations of sulfhydryl compounds in inducing cell division is interesting, since -SH compounds have frequently been suggested as being important in cell division.

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12. Cell Physiology

Peter Mazur

Peter Mazur
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12.1 SURVIVAL OF SUPERCOOLED YEAST

Peter Mazur R. H. Miller

As mentioned in the last Biology Division report,[1] even when yeast cells are cooled slowly enough to preclude intracellular freezing, 50% of the cells are still killed below −30°C. Some possible causes of death are the lowered temperature itself, the dehydration and concentration of solutes produced by ice formation, and interactions of solute concentration and reduced temperature.[2]

It is difficult to unravel the effects of these factors because a partially frozen solution has only one degree of freedom at constant pressure. Specifying the temperature, for example, determines the solute concentration and vice versa. However, this restriction is removed in supercooled solutions, and the present study is concerned with determining the effects of temperature and solute concentration on the survival of supercooled suspensions of yeast.

Cells of *Saccharomyces cerevisiae* were suspended in triple-distilled water or in 0.16 to 5 *m* NaCl solutions. Before use, the suspending media were filtered through 0.22-μ Millipore filters, and all glassware used to handle the cells and solutions was carefully rinsed in filtered triple-distilled water. With these precautions and by using small volumes (0.02 ml) of a dilute cell suspension (10^5 to 10^6 per ml), more than half of the cells in water could be supercooled to −18 to −20°C, and samples of cells in the NaCl solutions to −25°C or lower.

The usual procedure was to supercool the suspensions to −18 to −25°C for 10 to 180 min, warm them at defined rates, and compare the cell survival with that of frozen cells or cells that had remained at room temperature.

Cells suspended in pure water or in 2 *m* NaCl were not at all injured by being supercooled slowly or rapidly to −19°C for periods of time up to 2 hr. Survivals were 100% relative to room-temperature controls (Fig. 12.1.1).

The survivals of cells that froze at −20°C were much lower, but paradoxically they were higher (15%) for cells frozen in 2 *m* NaCl than for cells frozen in pure water (1%).

When cells were suspended in 3 to 5 *m* salt solutions, survival dropped rapidly with time even at room temperature. However, rapid supercooling of the suspensions to −25°C produced additional injury (Fig. 12.1.1).

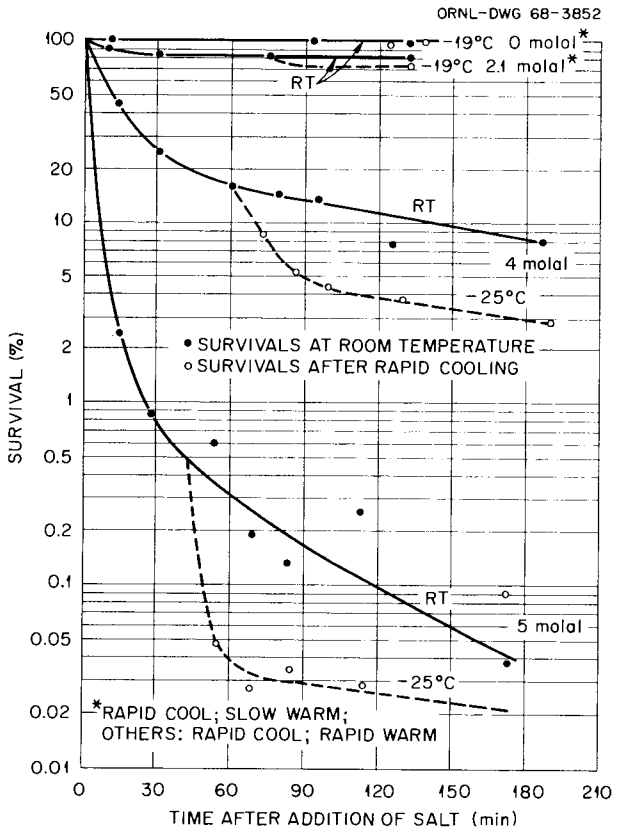

Fig. 12.1.1. Survival of Cells in Various Concentrations of NaCl as a Function of Time After the Addition of Salt. Cooling was initiated at the time indicated by the junction of the dashed and solid curves.

Cells *supercooled* to −25°C in 5 *m* NaCl were more injured than cells *frozen* in 2 *m* NaCl to −20°C, even though the concentration of NaCl in the unfrozen portion of the solution is about 5 *m* at −20°C. (The survivals were 0.03 and 15% respectively.)

The rapid drop in survival of cells held in concentrated salt solutions at room temperature (Fig. 12.1.1) made it difficult to evaluate the contribution of the lowered temperatures. Accordingly, the following experiment was performed. The cells were suspended in 0.16 *m* NaCl at room temperature (which is innocuous). The suspensions were then cooled to −3°C and a sufficient volume of 5 *m* NaCl at −3°C added to reduce the freezing point of the suspension to −3°C (the resulting concentration being 0.88 *m*). The suspension was then cooled to −6°C and sufficient 5 *m* NaCl at −6°C added to lower the freezing point to −6°C (the resulting concentration being 1.73 *m*). This stepwise cooling and addition of concentrated NaCl was continued until the samples had cooled to −18°C (still unfrozen). After 10 min at −18°C, the samples were warmed and simultaneously diluted back to 0.16 *m* NaCl. Parallel samples of cells in 0.16 *m* NaCl were caused to freeze at −3°C, then cooled in the same bath to −18°C, and finally thawed rapidly. In other words, the frozen samples underwent a smooth and progressive increase in salt concentration as cooling progressed. The unfrozen samples underwent a stepwise decrease in temperature and concomitant increase in concentration. The results of two experiments to date were surprising. The survival of the frozen cells was two to five times higher than that of the cooled but unfrozen cells.

The cause of the difference is unknown, but additional experiments have shown that it is not due to differences in the concentration of cells at the end of cooling.

The results for cells in water and 2 *M* NaCl indicate clearly that yeast cells are not susceptible to "thermal" shock (injury associated with rapid chilling) and, therefore, that thermal shock per se is not a factor in freezing injury. Brandts[3] has shown that the extent and rate of denaturation of proteins increase at temperatures below 0°C because of the anomalous thermal properties of hydrophobic bonds. Such denaturation apparently does not occur in 2 hr at −20°C in yeast in water or in 2 *m* NaCl, or else it is reversible or irrelevant to viability.

The interpretation of the findings for cells in concentrated NaCl is still unclear, especially the finding that cells in concentrated salt solutions are less damaged by freezing than by being cooled in the liquid state.

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12.2 NUCLEATION OF SUPERCOOLED SOLUTIONS OF NATIVE AND DENATURED MACROMOLECULES

Peter Mazur R. H. Miller

A number of investigators[1,2] have suggested that the water of hydration around native macromolecules is ordered and perhaps "icelike." This view is a matter of controversy, but even if not true there are strong reasons to believe that the nonpolar side chains of denatured proteins are surrounded by ordered water. So also may be the bases of denatured DNA. The reason for this belief is that hydrophobic interactions are thought to be responsible for 50 to 100% of the force stabilizing the native conformation of proteins,[3,4] and are believed by some[5] to be important contributors to the stability of DNA. Unlike most chemical bonds, the favorable free energy of the hydrophobic interaction comes not from a favorable enthalpy but from a favorable positive entropy change in the surrounding water structure associated with the transfer of a nonpolar side chain from water to the interior of the protein. The positive entropy reflects a decrease in the order of water structure. Conversely, the denatured protein is believed to have its nonpolar groups sticking into water, and the entropy decrease associated with this reaction is due to the formation of icelike structures around the nonpolar residues.

If macromolecules possess "icelike" hydration sheaths, and if these sheaths are sufficiently large and similar to normal ice, they ought to be able to nucleate supercooled water at higher temperatures than would otherwise be the case. The present work is concerned with testing this idea. Preliminary experiments to date have been concerned with the ability of native and denatured solutions of DNA to nucleate supercooled water. Control experiments have been performed with silver iodide.

A solution of 0.7 *M* NaCl was prepared and filtered through a 0.22-μ Millipore filter. Calf thymus DNA was added to a final concentration of 1 mg/ml. For an experiment, a 100-fold dilution was made using freshly triple-distilled water, and 0.02-ml quantities were transferred to small tubes. Half the tubes were heated to 90°C for 5 min and cooled in an ice bath to denature the DNA. The tubes, along with control tubes containing 0.02 ml of 0.007 *M* NaCl, were placed in a special ethanol-water bath and cooled at 1°C/min. The temperature at which each tube froze was noted, the temperature being measured with a quartz thermometer. All glassware was rinsed with the freshly triple-distilled water.

Silver iodide solutions were prepared by mixing together equal amounts of 0.01 *M* $AgNO_3$ and 0.01 *M* NaI. The resulting solution was diluted to 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} *M* using 10^{-5} *M* $AgNO_3$ as diluent to maintain the solution near its isoelectric point, and 0.02-ml quantities were transferred to supercooling tubes.

The mean freezing temperatures of the silver iodide solutions were −6.1, −9.5, −9.7, −12.7, and −13.4°C for the 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} *M* concentrations respectively. The water control froze at −23.6°C. The mean freezing point of the 0.007 *M* salt solution is −21.4°C so far. The means for the native and denatured DNA are −20.5 and −21.0°C.

Although the results with the DNA and salt solutions are not as yet complete and have not as yet been subjected to statistical analysis, they indicate that there is little or no influence of either native or denatured DNA on the extent of supercooling of dilute salt solutions. This would suggest that if there are icelike layers around DNA either they are very small (the nucleating ability of an ice crystal is proportional to its size) or they do not have the same structure as ice itself. The lack of effect from DNA contrasts with the clear nucleating ability of the dilute silver iodide solutions.

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12.3 EFFECT OF FREEZING ON THE SENSITIVITY OF CELLS TO TOXIC CHEMICALS

Steven Henikoff Peter Mazur
S. P. Leibo

The use of a number of agents in cancer chemotherapy is ruled out because concentrations high

enough to kill malignant tissue are also high enough to damage the host. An alternative approach to cancer therapy is cryosurgery. It involves destruction of malignant tissue by freezing it by means of a liquid-nitrogen-cooled probe placed within the tissue. The problem is that cells at the margin of the frozen tissue are likely to survive because the cooling velocity is not sufficiently high and the temperatures are not sufficiently low.[1] It seemed possible that the two approaches could be used to complement each other. The concentration of solutes in the unfrozen portion of a partly frozen solution is a function of temperature alone and is independent of the initial concentration. For example, the equilibrium concentration of sodium chloride in the unfrozen portion of a solution at −10°C is 2.64 *M*. This means that if a 0.16 *M* solution is frozen to −10°C, the concentration of sodium chloride will increase 16 times. So also will the concentration of any other solutes present in the solution. This suggested the possibility that cells could be placed at normal temperatures in a solution of a toxin sufficiently dilute to be innocuous, and then killed by being frozen to say −10°C, at which temperature they would have been subjected to a 16-fold increase in toxin concentration. Supporting the feasibility of this idea are a number of reports of chemical reaction rates being increased in partly frozen solutions,[2−4] presumably because of the mass action consequences of increased concentration. The idea was tested using yeast and human red blood cells and a variety of cell toxins. However, no significant effect was noted, a fact that has bearing on current theories of cell damage from freezing.

To be effective in this approach, the toxins should be sufficiently soluble at reduced temperature so that they do not precipitate out of solution before lethal concentrations are reached. Also, the dose-response curve should be sufficiently abrupt so that the increase in concentration produced by freezing will result in going from an innocuous concentration to a lethal one. A number of chemicals were screened in accordance with these requirements and the following selected for freezing studies: crystal violet, mercuric chloride, sodium hypochlorite, formaldehyde, phenol, lithium iodide, cupric chloride, sodium cyanide, lithium fluoride, cyclohexanone, iodoacetic acid, sodium azide, 2,4-dinitrophenol, methanol, hydrochloric acid, sodium hydroxide, Roccal, and cetylethylammonium bromide (CTAB).

Cells were suspended in 0.16 *M* concentrations of NaCl containing nonlethal concentrations of the toxin. They were then subjected to two freezing treatments.

1. Samples containing various concentrations of yeast cells were placed in the wells of multicompartmented plastic boxes, and 2% agar was added. The box and its contents were then cooled to −10°C at about 1°C/min, thawed and incubated at 30°C, and the number of colonies in each compartment was determined.
2. Samples were mixed with 15% gelatin and poured into flat-bottomed petri dishes. One end of a 1-in. copper probe was then inserted into the center of the petri dish, and the other end of the probe was placed in liquid nitrogen. The dish was cooled until the circle of frozen gelatin had expanded to within about 1 cm of the edge of the dish. The temperatures along a radius were monitored with thermocouples, and ranged from −60°C near the surface of the probe to 0°C at the margin of the circle of ice. The dish was then allowed to warm and thaw. Small plugs of gelatin at different distances along a radius were removed, dissolved in buffer, and assayed for survival. The assay for yeast survival was the standard dilution plate technique. The assay for red cell recovery was a colorimetric estimate of released hemoglobin.

No significant change in survival due to the presence of the toxic agents could be detected. There were suggestions of an increased lethality in a couple of cases, but the effect, if real, was marginal.

One of the current dogmas in cryobiology is that much of the lethality of freezing is due to the concentration of solutes by freezing. Our preliminary results are at variance with this idea, since freezing suspensions to temperatures at which the concentration of known toxins should have been increased to lethal levels did not produce increased lethality. The simplest hypothesis to account for the lack of effect would be that the reduction in reaction rate from the drop in temperature more than compensated for any increase in concentration produced by freezing. Other hypotheses are possible. For example, if the toxic effect were reversible, it

might be reversed when the toxin became diluted after thawing.

It still remains possible, of course, that an enhanced lethal effect can be demonstrated in other mammalian cells or with other toxins.

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12.4 FREEZING OF BACTERIOPHAGE T4B

S. P. Leibo Peter Mazur

We previously suggested that the inactivation of bacteriophage T4B by freezing and thawing may result from a competition for water of hydration between the phage and salts, or from an incompatibility between the structure of water ordered by the salt and that ordered by the phage.[1] Making use of observations previously presented in preliminary form,[1] the hypothesis has been substantiated.

Phage T4B is stable when suspended in and subsequently diluted from concentrated solutions of eight salts; but it is inactivated simply by suspension in concentrated solutions of seven different salts: NaBr, $NaClO_4$, NaI, LiCl, $MgCl_2$, $CaCl_2$, and $BaCl_2$. The inactivation does not appear to be related to salt concentration itself, ionic strength, osmotic pressure of the solution, or the individual ions in solution. But a correlation seems to hold when survival of the phage is plotted as a function of the water activity of the solution. Although the absolute value at which the phage is inactivated differs for different salts, in every case examined the inactivation occurs between a water activity of 0.75 and 0.95. Moreover, when phage survival is plotted in this way, the differences between salts suggest an ordering for effectiveness of both cations and anions to inactivate phage. The order follows the classical Hofmeister series. It was also determined that the inactivation of T4B in concentrated salt solutions does not result from osmotic shock, since the osmotic-shock-resistant mutant $T4Bos_1$ behaves exactly like the shock-sensitive form.

To test the hypothesis of freezing damage in T4B, we suspended the phage in 1.0 *m* $MgCl_2$. The phage is stable in that salt at 1 *m* concentration, but is inactivated at higher concentrations of the salt at 25°C. The concentration of salt solutions is fixed at subzero temperatures and is independent of the initial concentration. We then cooled the phage to various subzero temperatures above the eutectic point of $MgCl_2$, that is, –33.5°C. From the phase diagram of $MgCl_2$, one can determine the salt concentration and therefore the water activity in these partially frozen solutions. The results in Fig. 12.4.1 show that the survival of T4B in $MgCl_2$ as a function of subzero temperature agrees well with the survival of the phage plotted as a function of $MgCl_2$ concen-

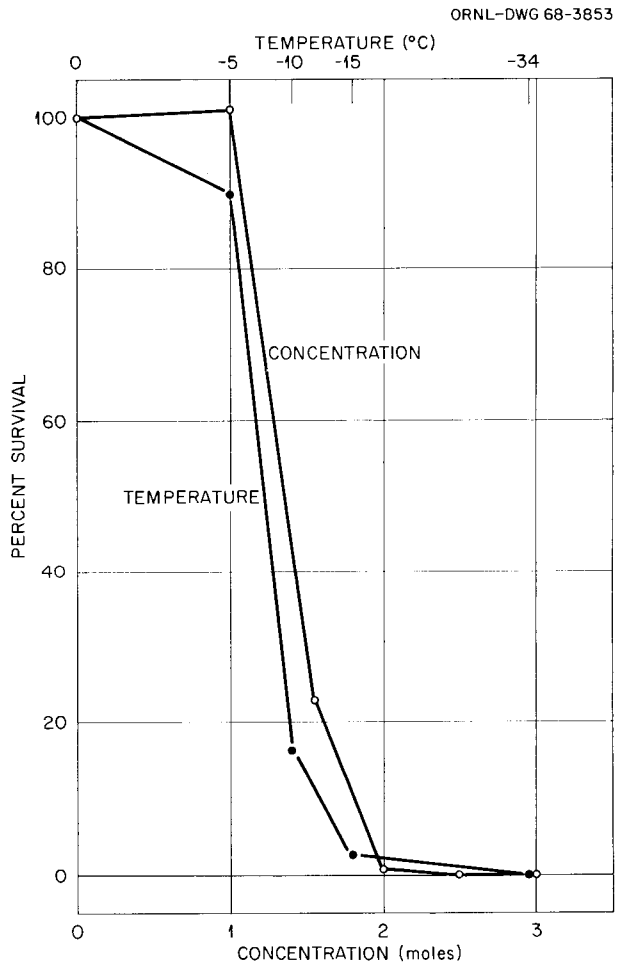

Fig. 12.4.1. Survival of Phage TyB as a Function of Subzero Temperatures and Concentration of $MgCl_2$.

tration at 25°C. (The results are presented in terms of salt concentration rather than water activity in this case to avoid the necessity for correction of water activity as a function of subzero temperatures.) In other words, phage T4B is inactivated at 25°C as the salt concentration increases, and the inactivation of T4B at subzero temperatures seems to result from the same increase in salt concentration as water is removed as ice. We believe that these data provide strong support for our hypothesis of freezing damage in bacteriophage T4B.

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12.5 OBSERVATIONS ON *os* MUTANTS OF BACTERIOPHAGE T4

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Wild-type strains of bacteriophage T4 are sensitive to osmotic shock when rapidly diluted.[3] Mutants resistant to osmotic shock (*os*) have been isolated.[4] All attempts to determine the chemical basis of osmotic shock resistance, however, have been unsuccessful.[5]

Using gel electrophoresis we have also attempted such a determination. This work was part of a continuing effort to determine the mechanisms by which the normal phage head is assembled.[6] We hoped that demonstration of a chemical difference between *os* and wild-type phage capsids would help to identify the product of another gene whose function is known to be essential for the production of normal phage heads. Unfortunately, we were not able to find such a difference.

The experiments were of three types: genetic, physical, and chemical. We prepared high-titer stocks of phages T4B and T4D in the presence of 5-bromodeoxyuridine, a potent mutagen. By means of three successive osmotic shocks, we obtained six independent isolates of both T4Bos and T4Dos. All further work was done with only one *os* mutant of each strain.

Mixed infections of *E. coli* with different ratios of T4D and T4Dos demonstrated that the yield of phages that are phenotypically *os* is directly proportional to the input ratio. Furthermore, when the phage yield was subjected to osmotic shock, the fraction of *genotypically* resistant phage did not change. This demonstrates phenotypic mixing for the *os* system. We then attempted to determine whether the *os* mutation is located within one of the known T4 genes by performing mixed infections of a restrictive bacterial host with T4Dos and each of five different *amber* mutants. The function of the genes defined by these *am* mutations is known to be essential for normal phage head production. The rationale of these experiments was that, if the *os* mutation is located on any of the genes possessing an *amber* mutation, only *os* phages would be produced in the restrictive host. Preliminary results indicate that the *os* mutation is located on gene 24. As part of these experiments we also isolated a T4Dos am_{24} double mutant.

Previous work had demonstrated that T4Bos has a higher density than that of T4B when centrifuged in CsCl gradients.[3] We have now found a similar relationship between T4Dos and T4D. Moreover, these four phages can be ordered in terms of increasing density in CsCl: T4D > T4B > T4Dos > T4Bos.

Using the disk electrophoresis procedure of Kellenberger,[6] we examined the banding patterns of the ghosts of phages T4B, T4Bos, T4D, and T4Dos. Under all conditions (CH_3COOH or NaOH degradation; electrophoresis at either pH 5 or pH 8), the electropherograms of these four phages were identical. Thus the molecular basis of osmotic shock resistance in T4 phage remains unknown.

Portions of this work were performed while S. P. Leibo was at the Institute of Molecular Biology, University of Geneva, Geneva, Switzerland.

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13. Chemical Protection and Enzyme Catalysis

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13.1 CHEMICAL PROTECTION

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The radiation LD_{50} method has proven to be a valuable one for the quantitative assessment of activity of chemical protective agents. However, little use of this procedure has been made in the recovery from radiation damage using bone marrow therapy. Various estimates of the number of marrow cells required for recovery from a given radiation dose have been made, but no systematic cell dose vs radiation dose response study has been reported. This study was undertaken to establish an optimal cell dose for supralethal radiation doses, to determine whether there was any significant secondary mortality by 90 days, and to examine the effects of large cell doses on intestinal recovery. Male and female (C57BL/Cum × C3H/Anf Cum)F_1 mice 10 to 12 weeks old were exposed in groups of ten to x-ray dosages in 75-r increments from 600 to 1650 r at a dose rate of 150 to 170 r/min. Syngeneic nucleated marrow cell suspensions were prepared from 6- to 10-week-old mice, and appropriate dilutions were made and injected intravenously via the tail vein one day after radiation. The mice were housed five per pan, and the mortality was recorded for 90 days. The 7-, 30-, and 90-day LD_{50} values were calculated by a probit analysis of the mortality data.

Since there was no significant difference in response between the males and females, the results were pooled for all experiments. The LD_{50} values obtained are presented in Table 13.1.1. It can be readily seen that a very small number of cells, for example, 2.5×10^4, has a significant effect on survival of the mice. There is a rapid rise in $LD_{50/30}$ with increasing cell dose up to 2×10^5 cells, which yield an increment of 467 r. Increasing the cell dose 1000-fold over this value produces a further increase of only 230 r. There thus seems to be a biphasic response, and in both phases the effect observed is proportional to the log of the number of cells administered. This response suggests that there is a relatively efficient seeding of the marrow with low cell doses and, over 1200 r, an inefficient and perhaps abscopal effect on intestinal damage in the mice. If the $LD_{50/7}$ is taken as a measure of intestinal damage, it can be seen from Table 13.1.1 that low cell doses have little effect but that doses of 5×10^6 cells and larger significantly raise the LD_{50} value. The 90-day LD_{50} was only slightly lower than the 30-day value at all cell and radiation doses. Even at low cell doses there was no indication of secondary disease or a late wave of

Table 13.1.1. $LD_{50/7}$, $LD_{50/30}$, and $LD_{50/90}$ Values for Various Nucleated Cell Doses

Cell Dose	Total Number of Mice	$LD_{50/7}$	$LD_{50/30}$	$LD_{50/90}$
0	1000	1320	753 ±6	753 ±6
2.5×10^4	850		820 ±12	810 ±10
5×10^4	448		853 ±11	822 ±10
1×10^5	459	1275	1043 ±11	995 ±12
2×10^5	800	1300	1220 ±15	1175 ±18
5×10^5	330	1360	1271 ±12	1220 ±10
2×10^6	220	1340	1279 ±32	1233 ±25
5×10^6	190	1430	1402 ±26	1329 ±20
1×10^7	220	1410	1364 ±46	1308 ±43
5×10^7	596	1500	1400 ±28	1365 ±27
1×10^8	270	1500	1440 ±40	1365 ±35
2×10^8	40		1450 ±35	1380 ±40

mortality, as has been previously reported. These results suggest that an optimum bone marrow cell dose for survival at high radiation doses is 5×10^6 cells.

The search for more effective and less toxic chemicals to protect against radiation damage has continued, and some 40 compounds of various types have been synthesized and tested. However, none of them has been as active as the well-known protective agents, and even in combination they have not offered any significant improvement.

13.2 STUDIES ON THE SUBSTRATE SPECIFICITY OF PEPSIN

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Margaret A. Turner

Previous studies on the specificity and kinetics of pepsin-catalyzed reactions using synthetic substrates have employed *N*-acetyl-, *N*-carbobenzoxy-, histidyl-, or *N*-glycyl di- or tripeptides or their ester derivatives as substrates. Furthermore, it was recently reported that the Michaelis constants for acetyl- and carbobenzoxy-L-phenylalanyl-L-phenylalanine differed considerably. This finding suggested that *N*-acyl groups other than amino acid residues may have an appreciable effect on the binding and kinetic parameters as certain dipeptide derivatives. To explore this hypothesis, we have synthesized and tested a series of derivatives of L-phenylalanyl-L-phenylalanine, X-L-Phe-L-PheCOY, in which X corresponds to benzoyl-, formyl-, isonicotinoyl, nicotinoyl-, or picolinoyl-, and Y corresponds to $-OH$, $-NHNH_2$, or $-OC_2H_5$. In addition, further modifications of various peptide-like compounds were synthesized and tested.

The rates of enzymic hydrolysis of isonicotinoyl-, nicotinoyl-, and picolinoyl-L-phenylalanyl-L-phenylalanine ethyl ester differed markedly, indicating that the position of the heterocyclic nitrogen has an appreciable effect on the susceptibility to pepsin hydrolysis. A kinetics study of hydrolysis of nicotinoyl- and isonicotinoyl-L-phenylalanyl-L-phenylalanine ethyl ester resulted in the following constants: $K_m = (1.84 \pm 0.06) \times 10^{-4}$ *M*, $k_3 = (13.6 \pm 0.8)$ min^{-1} and $K_m = 2.78 \times 10^{-4}$ *M*, $k_3 = 8.93$ min^{-1}. These data clearly indicate that the position of the heterocyclic nitrogen does indeed affect the kinetic parameters.

Table 13.2.1 presents hydrolysis data for our most rapidly cleaved substrates. It is apparent from these data that there is a striking difference in the kinetic parameters of picolinoyl dipeptide ester as compared with nicotinoyl or isonicotinoyl dipeptide ester. The corresponding free acids and hydrazides were cleaved at a reduced rate. In addition, of the four diastereoisomers of nicotinoyl-L-phenylalanyl-L-phenylalanine ethyl

Table 13.2.1. Hydrolysis Data for X-L-Phe-L-Phe $CO_2C_2H_5$ Series

X	pH	$[S]_0 \times 10^4$ (*M*)	$[E] \times 10^6$ (*M*)	$V_0 \times 10^6$ (*M*/min)
Formyl	3.80	1.50	3.21	(0.76)[a]
Nicotinoyl	3.80	1.50	3.21	(11.23); 18.80
Isonicotinoyl	3.80	1.50	3.21	10.02
Picolinoyl	3.80	1.50	3.21	(0.86)

[a]These measurements were conducted in the presence of 4% methanol.

ester, only the L-L isomer was hydrolyzed. We have also synthesized and tested the following compounds which were not hydrolyzed by the enzyme: acetyl-β-cyclohexylalanyl-L-phenylalanine, acetyldehydrophenylalanyl-L-phenylalanine, nicotinoyl-L-phenylalanyl-phenylserine, nicotinoyl-L-phenylalanyl-β-phenylethyl amide, and phenylpropionyl-L-phenylalanine ethyl ester.

It is of interest to note that increased susceptibility to pepsin hydrolysis is obtained only when the *N*-acyl group carries a positive charge in an aromatic ring system. In addition, conversion of one phenyl ring to cyclohexyl, for example, acetylcyclohexyl-L-alanyl-L-phenylalanine, yields a peptide resistant to pepsin attack. The rigid steric requirements, as well as the lack of hydrolysis of nicotinoyl-L-phenylalanyl-phenylethyl amide, indicate that there must be binding between the enzyme and substrate at three points on both sides of the susceptible bond.

14. Pathology and Physiology

Section Chief – A. C. Upton[a]

Pathological Effects of Neutrons, High-Energy Radiation, and Chemicals (14.1–14.7)

N. K. Clapp[a]
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E. B. Darden, Jr.[a]
W. H. Benedict[b]
K. W. Christenberry[b]
J. L. Montour[a,c]
J. C. Smith[b]
Niel Wald[b]
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J. W. Conklin[a]
M. L. Davis[d]
Mildred G. Hayes
M. C. Jernigan[a]
Lois H. Jordan
Faye S. Martin
Lou C. Satterfield
H. C. Swann[a]
V. K. Jenkins[d]

Cellular Pathology (14.8–14.11)

R. C. Brown
K. W. Cole
Florence N. Woodiel
Marion D. Bloomer

Physiological Effects of Radiation and Chemicals (14.12–14.14)

T. T. Odell, Jr.
C. W. Jackson
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Deborah E. Charsha[e]

Gnotobiotics (14.15–14.17)

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Virology (14.18)

R. L. Tyndall
N. D. Bowles
J. A. Otten
J. R. Parsons
Ernestine Teeter

Epidemiology and Microbiology (14.19–14.21)

R. C. Allen
J. H. Chandler, Jr.
G. W. Fortner
Jacqueline C. Holloway
R. T. Jones
Dorothy J. Moore
F. S. Shults
E. A. Smith
Birgitta Ruth Kullgren

Laboratory Animal Research and Technology		Histology and Autoradiography	
L. J. Serrano	W. B. Anthony	W. D. Gude	Joyce G. Freezell
J. B. Jones	T. R. Brown		Mary S. Gude
	N. L. Ensor		D. A. Jones
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	T. E. Lowery		Linda E. Duguay[e]
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	J. W. Cox		
	B. R. Wilson	*Participation in Cooperative Programs:*	
	R. A. Albertson	AEC-NCI Cocarcinogenesis (26.11–26.13)	
	Violet D. Black	AEC-NASA Space Biology (26.54, 26.55)	
	Emily L. Gaines		
	Josephine J. Lankford		
	J. R. Parsons		
	Phyllis L. Rouse		
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14.1 LOW (MEDIUM) LEVEL EXPERIMENT

All Members of Pathology-Physiology Section

The primary objective of this experiment is to determine the dose-response relation for radiation-induced carcinogenesis and life shortening under conditions of low-level irradiation in the mouse. It is also the aim of the experiment to obtain correlative data on the biochemical, physiological, microbiological, and morphological changes associated with late somatic effects of radiation and aging.

Previous progress reports have described the protocol of the experiment, the controlled environment in which experimental mice will be maintained, methods for detecting changes in the selected indigenous bacteria of the mouse intestine, the continuous absence of conventional mouse viruses, preliminary results with respect to longevity and incidence of leukemia, and a method for electromechanically recording and retrieving breeding data and preexperimental information for each mouse.

Results of preliminary studies were sufficiently definitive by August 1967 to verify that the protocol, procedures, and facilities for the experiment were satisfactory. Hence, irradiation of mice for the main experiment was begun at that time and has continued since then. Each week 120 mice in 15 cages, including unirradiated controls, are added to the experiment according to the plan shown in Table 14.1.1. The total number of mice to be set up has been reduced from about 40,000 of each sex to a total of 15,000 females alone, owing to fiscal limitations. This has involved, in addition to the omission of males, omission of groups exposed at low intensities and increase in the lowest radiation dose to be tested from 5 rads to 10 rads. During the past year, computer programs for storing, retrieving, and analyzing pathology and hematology data have been developed to complement those used for handling mouse ancestry, production, treatment, and life-history data. A small number of experimental mice has already died or been sacrificed, but data on pathologic effects in these animals are too few to warrant reporting at this time.

Table 14.1.1. Revised Radiation Dose Schedule for the Low (Medium) Level Experiment

Dose (rads)[a]	Total Number of Mice To Be Irradiated	Number of Mice Irradiated Each Week[b]
0	4000[c]	32
10	3000	24
25	1000	8
50	1000	8
100	1000	8
150	1000	8
300	4000[c]	32
Total	15,000	120

[a]Whole-body ^{137}Cs gamma rays, ~50 rads/min.

[b]Mice set up in groups of eight per cage.

[c]Number includes mice for serial sacrifice (only ~500 mice will be killed, but ~4000 need to be set up to provide survivors for sacrifice at intervals up to age of 30 months; the remaining ~3500 will die naturally and are thus comparable with the mice of all other groups).

14.2 PRELIMINARY OBSERVATIONS ON THE MODIFICATION OF LATE SOMATIC EFFECTS OF RADIATION BY HYPOXIA

E. B. Darden, Jr. K. W. Christenberry
M. L. Davis G. E. Cosgrove
M. C. Jernigan J. W. Conklin

Reducing the tissue oxygen concentration during irradiation has been shown to decrease susceptibility to a variety of early radiation effects, but less is known about its influence on susceptibility to induction of late somatic changes. We, therefore, have studied the influence of hypoxia on susceptibility to late pathological effects, particularly the induction of leukemias, other neoplasms, lens opacities, and life shortening. For this purpose, irradiation was carried out in a hypoxic atmosphere of 5% oxygen in helium, in a control atmosphere of 21% oxygen in helium, and in air. Helium was chosen for the inert component of the hypoxic gas mixture on the basis of the successful use of a similar mixture by Russell and colleagues some years ago in mice.[1]

Prior to each radiation exposure of mice in the gas chamber, we routinely allowed at least 15 min for bringing down the oxygen concentration and replacing the air with the proper mixture. Irradiation was not begun until the O_2 level had stabilized for an additional minute. This procedure was adopted after preliminary experiments had shown that the oxygen tension in vivo (as measured by a pO_2 needle electrode inserted intraperitoneally) responded more or less linearly in less than a minute to changes in the ambient oxygen concentration and that at the 5% O_2 level, which was the approximate threshold for survival, the pO_2 equilibrated at $\lesssim$10% of the normal value (~60 mm Hg). In orientation studies, we found that hypoxia increased the LD 50/30 for ten-week-old RF females to 1200–1300 r, or about double the value in air, while 30-day survival was increased from zero to ~90% at the 900-r level. For the main study, 13 replicate groups of mice received a single 300-kvp x-ray exposure at 90 r/min inside a Lucite flow-through gas chamber. An oxygen electrode inside the chamber monitored the ambient O_2 level continuously. After irradiation, mice in the various groups were examined periodically with a slit lamp for lens changes, and blood samples were taken periodically from the tail vein for hematological studies. At death, necropsies were performed, and histological specimens examined as needed.

With more than half of the 1371 30-day survivors dead in all groups, the data indicate that life shortening was substantially reduced in the hypoxia-treated groups, more than twice as high a dose being required to induce a given response in hypoxia as in air (Fig. 14.2.1). A small but consistent difference between the two nonhypoxic sets of mice in life shortening was also seen, suggesting that helium caused a slight increase in longevity. Preliminary results on the induction of leukemia and lens opacities, on the other hand, suggest that hypoxia had a smaller effect, if any, on the production of these diseases, but quantitative comparisons must await further analysis of microscopic, hematologic, and other data.

Reference

[1]W. L. Russell, H. C. Kile, Jr., and Liane B. Russell, *Genetics* **36**, 574 (1951).

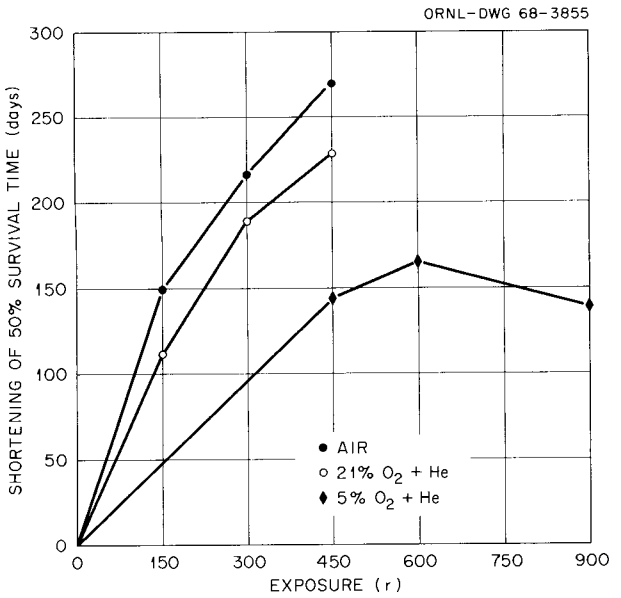

Fig. 14.2.1. Life Shortening in Relation to Dose, as Influenced by Hypoxia.

14.3 EVOLUTION OF CHROMOSOME ABERRATIONS IN RADIATION-INDUCED AND PASSAGED MOUSE GRANULOCYTIC LEUKEMIA

Niel Wald[1] S. Pan[1]
A. C. Upton R. C. Brown

A consistent secondary-constriction marker and an extra chromosome were described in RF mice developing chronic granulocytic leukemia after inoculation with the 21st to 27th serial passages of cellular or cell-free material from a post-x-irradiated leukemic primary mouse, or the 7th passage from a neutron-irradiated one.[2] Changes on further serial passages included the alternative appearance of a metacentric marker with no extra chromosome and finally no marker as the clinico-pathological nature of the originally chronic leukemia became increasingly acute. To examine this evolution further, cytogenetic and clinico-pathologic studies were performed on new primary post-x-irradiated leukemic mice and on the post-inoculation leukemic animals of successfully passaged lines.

Forty-one new primary granulocytic leukemic mice were acceptable for analysis. Twenty had a major or minor (5 to 10%) mode of cells with 41 chromosomes, ten a mode of 39, and eight had both. Fifteen had additional minute fragments, and nineteen showed gaps or breaks. Marker chromosomes of the secondary-constriction type were found in five, metacentric type in four, and giant type in four mice. Material from a primary with 41 chromosomes and a secondary-constriction marker has now undergone 13 successive passages. A modal transition from 41 through a 39/41 bimodal stage to a consistent 39 chromosome mode in the later passages, with successive modification and loss of the marker, was observed.

The results of these studies indicate that karyotypes of postirradiation primary chronic granulocytic leukemia in mice may present variable modal numbers and chromosome markers, which may be unstable on serial passage. These findings contrast with the rather consistent marker chromosome (phi chromosome) found in chronic granulocytic leukemia in man. Studies under way, including larger numbers of animals, will better define karyotype alterations in primary irradiation granulocytic leukemia in mice and the chromosome mode and marker transitions observed.

References

[1]University of Pittsburgh, Pittsburgh, Pa.
[2]Niel Wald *et al.*, *Science* **143**, 810 (1964).

14.4 RESPONSIVENESS OF LEUKEMIC CELLS TO GROWTH-REGULATING FACTORS

A. C. Upton Lou C. Satterfield

This experiment is part of a study aimed at exploring the extent to which leukocytosis-promoting factors may increase susceptibility to induction of granulocytic leukemia by irradiation, chemicals, or viruses and may stimulate the growth of leukemic cells of the granulocytopoietic series. The leukocytosis-promoting agent used in this work was a serially transplanted, slowly growing BALB/c mouse myoepithelioma,[1] which characteristically elicits marked granulocytosis in the blood of host animals. The effects of this neoplasm on the growth of a serially transplanted granulocytic leukemia which originated in an irradiated RFM/Un mouse are reported in the following.

The growth of transplanted granulocytic leukemia cells was appreciably accelerated in the presence

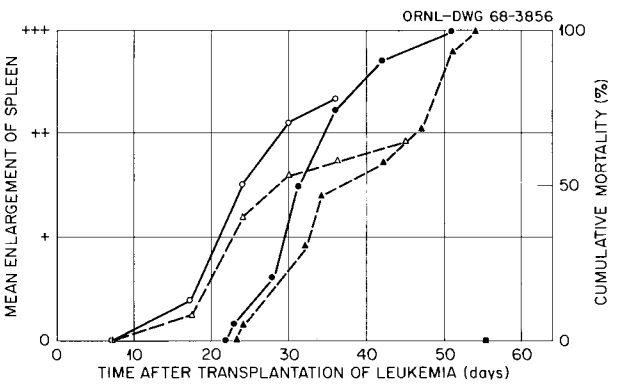

Fig. 14.4.1. Time Required for Spleen Enlargement and Death from Transplanted Leukemia, as Influenced by the Presence of Grafted Myoepithelioma. The recipients were young adult (BALB/c × RFM)F_1 mice. Shaded symbols denote data for cumulative mortality from leukemia; open symbols denote data for spleen enlargement. ○ = Recipients grafted simultaneously with myoepithelioma subcutaneously and 10^6 leukemia cells intravenously (20 mice). Δ = Recipients grafted intravenously with 10^6 leukemia cells alone (19 mice). □ = Recipients grafted subcutaneously with myoepithelioma alone (25 mice).

of the myoepithelioma, as judged by the time required for the leukemia cells to cause splenomegaly and death in syngeneic recipients (Fig. 14.4.1). In contrast, the myoepithelioma had no such effect on the growth of syngeneic lymphoma cells transplanted into similar recipients. These results imply that the growth-stimulating effects of the myoepithelioma resulted from its leukocytosis-promoting action, possibly via leukopoietin, rather than from any nonspecific impairment of host resistance. This interpretation remains to be verified, however, by more-direct analysis of effects on the rate of proliferation of the leukemic cells themselves. Such experiments, with the use of tritiated thymidine labeling of mitoses as a means for measuring the duration of the cell cycle, are in progress.

Reference

[1]The authors are grateful to Thelma Dunn of the National Cancer Institute for kindly supplying this tumor.

14.5 HEMOPOIESIS IN IRRADIATED RFM MICE AS STUDIED BY THE SPLEEN COLONY ASSAY

V. K. Jenkins A. C. Upton
T. T. Odell, Jr.

A technique widely used for studying hemopoiesis is derived from the observation of Till and McCulloch that normal hemopoietic tissue includes cells capable of rapid colonization in the spleens of irradiated recipient mice.[1] Another technique for obtaining a quantitative measure of the proliferative capacity of marrow is to evaluate the colonies developing from endogenous stem cells in spleens of mice exposed to sublethal irradiation. This report concerns the relative proportions of spleen colonies of different hematologic types produced in RFM mice by these two techniques.

Spleens of RFM mice exposed to 400–500 r and killed six to eight days later had a high proportion of myelocytic colonies and relatively few erythropoietic colonies (Fig. 14.5.1). The total numbers of colonies and the number of each histologic type decreased with increasing radiation dose, a result consistent with the expectation that increasing irradiation would kill increasing numbers of the hemopoietic stem cells from which the colonies were derived. The proportions of endogenous colonies were time dependent: the numbers of myelocytic colonies decreased between six and nine days after irradiation, whereas numbers of erythropoietic colonies did not change with time. Mice exposed to 750 r of x rays, injected with syngeneic bone marrow, and killed six to nine days later had spleen colonies which were predominantly erythropoietic and undifferentiated (Fig. 14.5.1). The numbers of colonies were approximately a linear function of the numbers of exogenous marrow cells injected, one colony being produced by 2×10^4 exogenous cells. The numbers of spleen colonies remained fairly constant over the four-day period studied, and the relative proportions of different types of colonies were consistent with those reported for other strains of mice.

The reason for the differences between the endogenous and exogenous systems in the relative proportions of colonies of different cytologic types is not known. One possibility is that the mechanisms that control some or all types of hemopoiesis are damaged by irradiation, with the result that the growth and differentiation of colony-forming cells may be different in mice exposed to

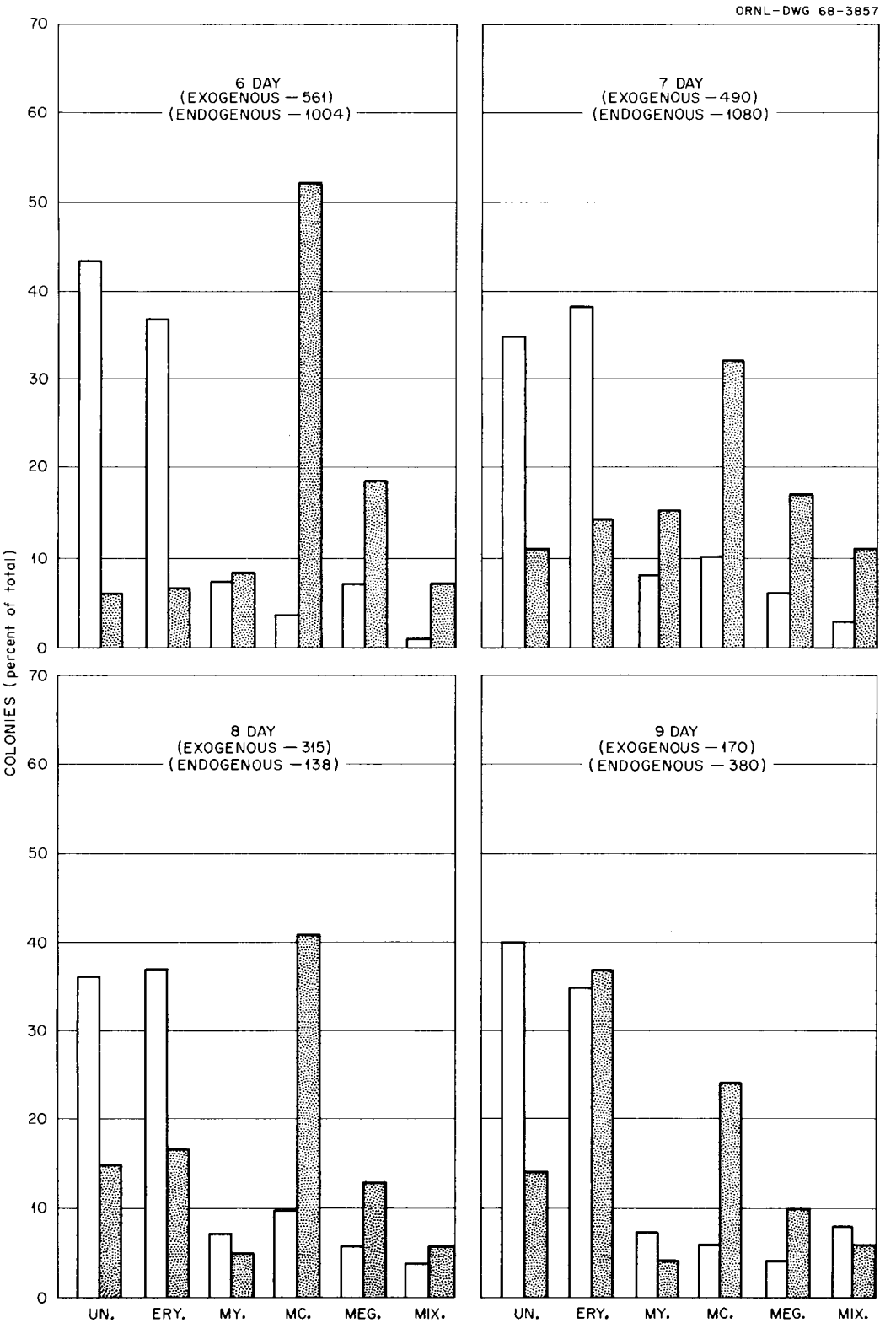

Fig. 14.5.1. Percentages of Colony Types Produced from Endogenous and Exogenous Marrow Cells in Female Mice Sacrificed Six to Nine Days After Irradiation or Marrow Injection. Open bars indicate percent for exogenous and shaded bars give percent for endogenous cells. Total numbers of colonies examined are given in parentheses. Abbreviations are: Un. — undifferentiated, Ery. — erythropoietic, My. — myeloid, Mc. — myelocytic, Meg. — megakaryocytic, and Mix. — mixed.

lethal levels of radiation than in those exposed to sublethal doses. Another possible explanation is that the transplanted stem cells are damaged by handling or the endogenous cells by irradiation, with the result that the proportions of cells remaining viable and their ability to differentiate may be impaired differentially in the two situations.

Other investigators have reported that mice of strains other than RFM have endogenous spleen colonies with a high proportion (~75%) of erythropoietic and a low (0 to 9%) proportion of granulopoietic types.[2] One possible explanation for this difference in proportions of colony types between RFM mice and mice of most other strains is that extramedullary myelopoiesis is greater in RFM mice than in mice of the other strains.

References

[1] J. E. Till and E. A. McCulloch, *Radiation Res.* **14**, 213–22 (1961).

[2] J. L. Curry, J. J. Trentin, and N. Wolf, *J. Exptl. Med.* **125**, 703–20 (1966).

14.6 EFFECT OF ESTRADIOL ON HEMOPOIESIS IN RFM MICE

V. K. Jenkins A. C. Upton
T. T. Odell, Jr.

In RFM mice the effect of estradiol treatment on repopulation of spleen and bone marrow by endogenous hemopoietic cells or exogenous bone marrow cells was studied by scoring numbers and cytologic types of colonies and/or measurement of ^{125}IUdR (DNA synthesis) and ^{59}Fe (erythropoiesis) uptake by spleen and bone marrow.

Endogenous hemopoietic spleen colonies developing in mice of both sexes were predominantly of the myelocytic type six to seven days after 425 to 475 r of x irradiation. The total number of endogenous colonies decreased with time after irradiation, owing principally to a decrease in the numbers of myelocytic colonies. Estradiol treatment drastically reduced the number of myelocytic colonies (Table 14.6.1) and correspondingly increased the numbers of colonies of undifferentiated and erythropoietic types. Isotope uptake data confirmed the estradiol-induced increase in erythropoiesis. Estradiol treatment also prevented the time-dependent decrease in total colony numbers, probably by increasing the relative numbers of undifferentiated and erythropoietic colonies and decreasing the relative number of myelocytic colonies from the start.

The spleen colonies in lethally irradiated mice injected with 1×10^5 syngeneic marrow cells were predominantly of the erythropoietic and undifferentiated (70 to 80%) types, with less than 10% being of the myelocytic type. Estradiol treatment had no appreciable effect on the number or types of these exogenously derived colonies or on the uptake of ^{125}IUdR or ^{59}Fe by the spleen during the six- to nine-day period. Estradiol treatment of bone marrow donors, however, resulted in a significant decrease in numbers of CFU in their marrow and in the ability of their marrow to promote radioactive isotope uptake in recipient mice.

The data imply that estradiol treatment reduces the numbers of stem cells in the marrow cavity and that it depresses myelopoiesis and increases erythropoiesis in the spleen of the sublethally irradiated mouse. The mechanism of action of estradiol on hemopoietic cells has not been clarified. Although estradiol may indirectly force CFU from the marrow cavity by stimulating endosteal bone formation, a direct effect of the hormone upon the stem cells is also suggested.

14.7 INFLUENCE OF AGE ON HEMOPOIETIC REPOPULATION OF THE SPLEEN AFTER WHOLE-BODY IRRADIATION IN RFM MICE

M. L. Davis A. C. Upton
G. E. Cosgrove

This study was undertaken to investigate the mechanism of previously observed age-related variations in the susceptibility of mice to induction of leukemia by irradiation. To approach this question, we examined the influence of animal age on recovery of hemopoietic cells after irradiation, as judged by the cytologic pattern of repopulation of the spleen.

Female RFM mice were exposed at 2 to 17 months of age to 750 r whole-body x radiation, after which they were promptly injected with 10^4 to 10^6 isologous nucleated bone marrow cells from 14-week-old donors. Six days later they were sacrificed and their spleens removed for histologic examination. The numbers and types of hemopoietic colonies observable microscopically in a midline

Table 14.6.1. Comparison of Mean Numbers of Colonies per Spleen in Sublethally Irradiated Mice Treated with Sesame Oil and Estradiol[a]

Day Killed	Sex	Number of Mice[b]	Mean Numbers of Colonies[c]							
			Total		Undifferentiated		Erythropoietic		Myelocytic	
			Sesame Oil	Estradiol	Sesame Oil	Estradiol	Sesame Oil	Estradiol	Sesame Oil	Estradiol
6	F	(41) (27)	20	20	1	3	2	10	11	1
	M	(21) (10)	23	20	1	1	4	8	11	2
7	F	(35) (36)	14	21	2	4	2	10	5	1
	M	(11) (9)	10	17	1	2	1	6	4	1
8	F	(15) (21)	9	17	1	3	2	10	4	1
	M	(18) (15)	6	13	1	3	2	6	2	1
9	F	(36) (16)	11	19	2	3	4	12	3	<1
	M	(33) (15)	7	14	1	3	2	8	2	<1

[a]Mice were eight weeks old at time of irradiation.

[b]The first number in parentheses indicates sesame-oil-treated mice and the second indicates estradiol-treated mice. The mice were given three to five intramuscular injections of estradiol (33 μg in 0.1 ml of sesame oil) or vehicle, beginning within 24 hr after irradiation and continuing every other day until the animal was killed.

[c]Mean numbers of colonies were rounded off to nearest whole number.

section of each spleen were then analyzed in relation to the age of the recipient. The results of this analysis showed little change with age except in the numbers of megakaryocytic colonies; these increased by an order of magnitude as the age of the recipient increased from 30 to 75 weeks; however, a comparable splenic megakaryocytosis was observed in a few similarly irradiated old mice, even in the absence of injected marrow cells. Hence it must be assumed that the megakaryocytes in the old mice may have been derived from host cells, as well as donor cells, although the extent to which they were of host, as opposed to donor, origin remains to be determined.

The megakaryocytosis in the spleens of aged irradiated mice points to an age-related change in the growth and differentiation of hemopoietic cells, which may be of significance in relation to homeostasis, leukemogenesis, and senescence. This possibility deserves to be explored further and is the basis for additional studies which are in progress.

14.8 X-RAY AND X-RAY-PLUS-URETHANE INDUCTION OF "LEUKEMOGENIC" ACTIVITY IN TISSUES OF MICE: ELECTRON MICROSCOPIC STUDIES

Vincenzo Covelli R. C. Brown
R. L. Tyndall

Haran-Ghera has reported a prompt appearance of leukemogenic activity in cell-free thymus–bone-marrow homogenates of C57BL mice soon after four weekly doses of x rays, or four weekly doses of x rays and urethane. Maximum leukemogenic activity, measured by injecting homogenates into intrarenal thymus grafts in isologous thymectomized irradiated mice, occurred in homogenates prepared from animals ten days after the last radiation dose. She postulated that x rays "activated or released" a leukemogenic substance, but she did not examine the homogenates for the presence of virus particles. In order to elucidate further whether virus-like agents might be released by x rays plus urethane, we performed qualitative ultrastructural examinations of cell-free homogenates and tissues of C57BL and RFM/U mice treated with x rays alone or x rays and urethane according to the methods of Haran-Ghera.[1] As positive controls for the presence of virus particles, Rauscher-virus-injected newborn BALB/c mice were also examined.

Thin-sectioned Epon-embedded preparations of glutaraldehyde-fixed, osmium-post-fixed thymus, bone marrow, and splenic tissues and cell-free pellets prepared from homogenates of either pooled thymus–bone-marrow tissues or pooled spleen tissues were examined with an HU 11B Hitachi electron microscope. The specimens were stained with uranyl acetate and lead citrate. The cell-free pellets were prepared by ultracentrifugation (48,000 $\times$ g for 30 min) of the supernatant[1] of homogenates of tissues pooled from 2 to 18 mice. An indefinable number of virus-like particles were found in cell-free pellet preparations from all groups of animals, whether treated mice or controls. The virus-like particles were round, contained a variably electron-dense nucleoid and well-defined outer unit-like membrane, and had a diameter of 800 to 1000 A – all consistent with the morphology of "murine leukemia viruses." On the other hand, no virus-like particles were found in the tissues of any control group, whereas in tissues of animals injected with Rauscher virus they were easily found in all three organs examined, and in one C57BL x-ray-plus-urethane-treated mouse, a few virus-like particles were found in sections of the thymus.

The presence of virus-like particles in the treated groups is consistent with Haran-Ghera's hypothesis of virus "release" but does not verify it. The presence of virus-like particles is not unexpected in untreated mice, since similar particles have been found by others in untreated mice of other strains.[2] That the tissue sections of C57BL and RFM/U mice contained few, if any, virus-like particles, whereas the Rauscher-virus-injected mice contained many particles, indicates the existence of a quantitative difference in numbers of contained virus-like particles, which was not reflected in the results we obtained on examination of pellets from tissue homogenates; however, our technique did not allow quantitation, due to nonrandom distribution of particles within a pellet, most of the particles being at the top of the pellet. Future experiments to quantitate the occurrence of virus-like particles in treated and untreated groups would help clarify the meaning of virus "activation." It would also be of interest to see the cell distribution of viruses after different types of "activation" and in different mouse strains.

References

[1]N. Haran-Ghera, *Intern. J. Cancer* **1**, 81–87 (1966).

[2]F. E. Leaders *et al.*, *Intern. J. Cancer* **2**, 365–69 (1967).

14.9 ULTRASTRUCTURAL STUDIES OF A HIGH-MORTALITY LAPINE ENTERITIS

R. C. Brown C. B. Richter[1]
M. D. Bloomer

Enteric disease associated with high mortality is common in wild rabbits brought into the laboratory environment. The disease has seriously interfered with attempts to conduct experimental studies using wild rabbits in many different laboratories, including the Oak Ridge National Laboratory, where studies using Shope papilloma virus are being conducted. Such a lapine enteritis causes up to 50% mortality in wild Kansas cottontail rabbits imported into the Oak Ridge National Laboratory. Most of the mortality occurs during the first two weeks of confinement. The disease begins with diarrhea, and death usually occurs within 12 to 24 hr later. Most of the animals die with spasticity, but some die in a "shocklike" state. The significant pathology, as observed by Richter, is a severe enteritis involving the cecum and colon, with less intense involvement of the terminal ileum. It is characterized by marked cecal dilatation, bowel edema and congestion, epithelial cell necrosis and exfoliation, and marked epithelial cell regenerative response. Characteristically, small focal gastric mucosal ulcerations occur, but no significant generalized necroses or gastritis is found. The etiology, pathogenesis, and means of preventing this disease have not been discovered despite extensive attempts to incriminate an infectious agent. In the hope of better understanding the disease, we have made comparative studies of the ultrastructure of the intestinal tract of clinically diseased animals and clinically well rabbits.

Ultrastructural changes were seen in the diseased bowel of the clinically sick wild rabbits that confirm structural changes seen by light microscopy. The major cellular pathology in the cecum, colon, and ileum consisted of nonspecific vacuolation and necrosis of epithelial cells, which was, in the less advanced disease, almost always confined to the cells of the macrovilli and not the gland cells or crypt epithelial cells; in the more advanced cases necrotic vacuolated epithelial cells were sloughed from the macrovilli and crypts into the bowel lumen, with only a few glands remaining intact. A variable degree of cellular edema was present in all layers of the bowel. Mitochondrial degeneration was present in some villous epithelial cells which showed no other detectable degenerative changes. Sparsity and shortening of microvilli of some of the epithelial cells was observed. The cecum and colon, as noted by light microscopy, showed the most pronounced pathology.

The clinically sick wild rabbits showed the presence of numerous virus-like particles on the surface of the epithelium of the intestine, within some cells of the necrotic gastric lesions, and within some epithelial cells lining the intestine. The virus-like particles were most numerous on the epithelial cell surface (on or between microvilli) of the cecum and colon and less numerous on the epithelial surface of the ileum, in the lumens of glands, and in the intestinal crypts. Conglomerations of particles were present in the mucus in the intestinal lumen but not on the surface of the gastric epithelium nor in the gastric mucus. The intracellular virus-like particles were usually within membrane-bound vesicles resembling lysosomes, but in necrotic cells they were found free in the cytoplasm. In the punctate gastric lesions, necrotic cells contained particles within cell cytoplasm, within mitochondria, and within membrane-bound vesicles. No particles were found budding from cell membranes. The site of particle production has not been definitely identified. The virus-like particles varied in diameter from 500 to 700 A and were rounded in profile. A single variably dense nucleoid, about 350 A in diameter, was present in about 25% of the particles on the intestinal epithelial surface and in usually more than half of the intravesicular and intracytoplasmic particles. The outer membrane of these particles had a trilaminar structure like that of the unit membrane. Since the virus-like particles were similar in size to the intracystic vesicles of multivesicular bodies, considerable care was needed to differentiate these when the intravesicular particles were without nucleoids.

Negative stain preparations of cecal surface material showed rounded 600- to 800-A-diam particles with no evidence of capsomeres. The outer double membrane was easily delineated in most particles. A small percentage of particles had solid core not penetrated by stain, which was interpreted as representing the nucleoid. Most particles were penetrated by variable amounts of stain centrally, but rather uniformly, and the central area was usually less dense than the

narrow zone between the apparent nucleoid and particle outer membrane.

The significance of these virus-like particles is uncertain since they were also found, although usually in fewer numbers, on the intestine epithelial cell surface of all the clinically well rabbits. Rarely, intravesicular particles were also found in intestinal and gastric epithelial cells of the clinically well rabbits; however, no cellular or tissue lesions were observed in these animals. Studies are presently under way to elucidate the nature and significance of these virus-like particles.

Reference

[1]Mammalian Recovery section.

14.10 CYTOGENETIC STUDIES OF HEMOPHILIA-LIKE DISEASE OF SWINE[1]

R. C. Brown K. W. Cole
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A hemophilia-like disease occurs in swine, which is characterized by a low plasma antihemophilic factor, prolonged coagulation time, prolonged bleeding time, and a recessive mode of inheritance. Serum and plasma transfusion experiments suggest that the disease is similar to von Willebrand's disease (vascular hemophilia) in man. Prolonged bleeding time, lack of sex-linked inheritance, and results of serum and plasma transfusion studies distinguish it from the classical hemophilia described in man and in dogs. Cytogenetic studies on swine with this hemophilia-like disease seemed pertinent in order to establish whether the disease was associated with an abnormality of karyotype.

The karyotypes of male and female hemophilic-like swine were compared with karyotypes of normal swine. The chromosome preparations were made from phytohemaglutinin-stimulated blood leukocytes and also from bone marrow cell cultures. The hemophilic-like swine had karyotypes consisting of 38 chromosomes, the morphology of which was indistinguishable from normal. These findings suggest that hemophilia-like disease in swine, like classical hemophilia in man and dogs, is probably due to a point mutation or to a submicroscopic chromosomal aberration and not to a microscopically detectable chromosomal change.

References

[1]This investigation was supported in part by PHS grants HE-01648, HE-06350, and HE-07181 from the National Heart Institute.

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[3]Department of Pathology, University of North Carolina, Chapel Hill.

14.11 THE DEVELOPMENT OF A RELIABLE GRAM STAIN FOR BACTERIA IN TISSUE SECTIONS[1]

R. C. Brown Howard C. Hopps[2]

The identification of bacteria in tissue sections is often of crucial importance in establishing the etiology of specific lesions, because there is no opportunity to apply conventional bacteriologic methods in arriving at a diagnosis. Difficulties with present bacterial stains have led to frequent use of a battery of them, with much expenditure of time and energy and, usually, with less than satisfactory results. We decided that the best approach to development of a reliable tissue Gram stain was first to evaluate some of the presently best known tissue Gram stains, and then to proceed to modify these, if possible, to achieve a consistently reliable procedure.

Brown-Brenn,[3] MacCallum-Goodpasture,[3] and Humberstone[4] tissue Gram stain methods were evaluated, using experimentally induced "standard" tissue lesions (skin lesions in mice) containing *E. coli* and *Staphylococcus albus*. The effects of varied types of fixatives (10% formalin, sodium acetate or sodium phosphate buffered, Formol-Zenker's fluid, Bouin's solution, and 2.5% glutaraldehyde, phosphate buffered) and duration of fixation (1 day or 60 days), types of paraffin (Bioloid and Paraplast), and section thickness (4 or 6 μ) were studied. Evaluation showed that the two principal difficulties with these stains were: (1) failure to stain Gram-negative bacteria and (2) failure to differentiate Gram-negative bacteria from background (stained) tissue.

Through systematic variations of reagents and procedures and by carefully controlled manipulations involving the processing and evaluation of numerous slides, we were able to develop a Gram staining method that clearly and consistently differentiates Gram-negative and Gram-positive

bacteria in tissue sections. This method, which was achieved by significantly modifying Humberstone's Gram stain procedure,[4] uses the following solutions: (1) aqueous crystal violet, 1% solution; (2) Gram's iodine; (3) Cellosolve (ethylene glycol monoethyl ether); (4) aqueous basic fuchsin, 0.5% solution; (5) Gallego's differentiator: distilled water, 50 ml; 40% formaldehyde, 1 ml; glacial acetic acid, 0.5 ml; and (6) aqueous tartrazine, 1.5% solution. The method is as follows: (1) Remove wax from paraffin sections with xylene, and bring sections through graded alcohols to distilled water; (2) stain in 1% aqueous crystal violet for 2 min; (3) rinse in distilled water; (4) mordant in Gram's iodine for 5 min; (5) rinse in distilled water; (6) differentiate in Cellosolve until blue color no longer streams away from the section (approximately 5 to 10 sec); (7) quickly wash with distilled water; (8) stain in basic fuchsin for 5 min; (9) wash in distilled water; (10) differentiate and "fix" basic fuchsin in Gallego's differentiator for 5 min; (11) wash *thoroughly* in distilled water; (12) blot lightly, only to remove excess water (not to dryness); (13) apply 1.5% aqueous tartrazine, and immediately blot away excess, but not to dryness; (14) quickly pass the slide through three changes of Cellosolve (six quick dips each); (15) pass through three changes of xylene, approximately ten quick dips each; the slide can then remain in the last xylene until convenient to mount; (16) mount in Permount. Steps 6, 7, 13, and 14 are critical in regard to timing. Preferably, all stains and solutions are applied to the slide in a horizontal position, except for steps 6, 14, and 15, in which case the slide is dipped into the solutions contained in Coplin jars.

This stain works satisfactorily on tissues fixed in 10% formalin (sodium phosphate or sodium acetate buffered), glutaraldehyde (2.5% in phosphate buffer), and Formol-Zenker's fluid. It does *not* work well on Bouin's fixed tissues. The method has been successfully used to demonstrate many different types of bacteria in tissue sections derived from human lesions, including *Staphylococcus albus, Hemophilus influenza, Pasturella pestis, Pseudomonas pseudomallei, Mycobacterium leprae,* and *Mycobacterium tuberculosis.*

References

[1]This research was supported in part by Army Medical R and D Grant 3A014501B71Q.

[2]Chief, Division of Geographic Pathology, Armed Forces Institute of Pathology, Washington, D.C.

[3]*Manual of Histologic and Special Staining Techniques*, 2d ed., Blakiston Division, McGraw-Hill, New York, 1960.

[4]F. D. Humberstone, *J. Lab. Med. Tech.* **20**, 153–56 (1963).

14.12 GENERATION CYCLE OF RAT MEGAKARYOCYTES

T. T. Odell, Jr. C. W. Jackson
Rebecca S. Reiter

To gain additional understanding of the maturation process of megakaryocytes we investigated the time parameters of their generation (endoreduplication) cycle, using the technique of determining the percentage of labeled mitotic figures at intervals after a single injection of tritiated thymidine, a specific DNA precursor. In addition, changes in the rate of synthesis of DNA on a per cell basis during the course of the synthesis period were studied by counting the number of grains over each labeled mitotic megakaryocyte at intervals after injection.

Bone marrow smears were examined at 12 time periods between 30 min and 12 hr after injection of ^{3}H-thymidine. A total of 1065 mitotic megakaryocytes were included in the study, and no evidence of cytokinesis was seen in any megakaryocytes. All mitotic megakaryocytes without differentiation into ploidy classes were included. Thirty minutes after injection none of the mitotic megakaryocytes was labeled, indicating that the cells that were labeled late in the period of DNA synthesis (S) had not yet passed through the post-synthetic, premitotic period, G_2. Forty-two percent were labeled at 1 hr and 100% at 2 hr. The labeling index remained at 100% through the seventh hour but dropped to 82% at 8 hr and 36% at 9 hr. After 10 hr the labeling index began to rise again. The duration of a complete generation cycle, determined by finding the length of time between the 50% labeling points on the first and second ascending curves, was 9.3 hr, and DNA synthesis time was 7.6 hr based on the difference in time between the 50% labeling points on the ascending and descending limbs of the labeling index curve. The relatively rectilinear nature of the labeling index curve indicated that the cycle does not vary greatly among megakaryocytes of different ploidy classes.

Since the number of grains per cell is a rough measure of the relative amount of ^{3}H-thymidine incorporated, the changing mean grain counts over megakaryocytes during the course of the S period indicate a changing rate of synthesis per cell. The results indicated that the DNA synthetic rate rises to a peak between the fourth and the fifth hour after the beginning of S and falls rapidly during about the fifth to the seventh hour.

14.13 ANALYSIS OF MICROSPECTROPHOTOMETRIC MEASUREMENTS OF DNA OF MEGAKARYOCYTES

D. G. Gosslee[1] T. T. Odell, Jr.
C. W. Jackson

Three methods for estimating the proportion of megakaryocytes in each of several DNA (ploidy) classes were compared in the previous progress report. Of the three, the maximum likelihood method was preferred because it gave the best estimates with the fewest restrictions; for example, it was not necessary to assume a doubling of DNA in successive ploidy classes. Nevertheless, in an attempt to understand the causes of the large variability in quantity of DNA among megakaryocytes assigned to the various ploidy classes, additional analyses of microspectrophotometric measurements of DNA in rat megakaryocytes were done. Various subpopulations were analyzed, and the results were compared.

Multiple measurements made on a set of megakaryocytes showed that the variation among cells of a ploidy class was about three times that among repeated measurements on the same megakaryocyte, indicating that variability in measurements on any particular cell contributed relatively little to the total amount of variation. In addition, variability in amount of DNA among cells of various ploidy classes was similar whether or not the populations included megakaryocytes in the process of synthesizing DNA. Other studies have shown that only about 7% of the total recognizable megakaryocyte population is in DNA synthesis at any one time. The presence of megakaryocytes in DNA synthesis does not appear therefore to add greatly to the variability. On the other hand, comparison of sets of megakaryocytes of the same ploidy classes but having different nuclear conformations did reveal differences in mean DNA values. For example, the mean DNA values of naked nuclei, the final megakaryocyte stage in the marrow, were consistently lower than those of megakaryocytes in mitosis. It is readily apparent upon viewing these nuclei through the microscope that the spatial distribution of DNA is quite different. Although the two-wavelength method of microspectrophotometry used in these studies greatly reduces the distributional error, the variability in relative density and depth (path length) of DNA within megakaryocyte nuclei is rather large and probably makes an appreciable contribution to the variability of DNA within ploidy classes observed in these studies. The failure of mean DNA values of successive ploidy classes to have exactly twice the mean value of the preceding class also implicates a measurement error due to DNA distribution.

Because of the several possible sources of variability mentioned here it does not seem reasonable to suggest an inconstancy of DNA among megakaryocytes as the explanation of the varying values of DNA within ploidy classes.

Reference

[1]Mathematics Division.

14.14 SIZE DISTRIBUTION OF BLOOD PLATELETS AND ERYTHROCYTES FOR 74 DAYS AFTER X IRRADIATION

T. T. Odell, Jr. R. S. Reiter
C. W. Jackson

The damaging effects of whole-body radiation on the hemopoietic system are well documented. Exposure to relatively large doses produces a severe aplasia of the marrow within a few days, and a consequent reduction in peripheral blood cells. In recovering animals, the age compositions of the circulating populations of cells change when newly formed cells enter the circulation because of increases in the proportions of young cells. Since it has previously been shown that young blood platelets and erythrocytes are larger than older ones, some aspects of the dynamics of recovery of the hemopoietic system from radiation can be investigated by studying changes in the size distribution of populations of peripheral blood cells.

Rats were exposed to approximately an $LD_{50/30}$ of x rays (950 r), and three to four were killed at frequent intervals up to 74 days for examination of platelet and erythrocyte number and size distribution, as well as megakaryocyte number in the marrow and reticulocyte count in the blood. (Cell size distribution was determined with a Coulter detector combined with a 400-channel analyzer.) There was essentially no measurable change in the size distribution of the platelet population during the first week after exposure to radiation, when the number of cells in the peripheral blood was declining. On the 11th day after radiation, the next time when there was a sufficient number of platelets present to obtain a sample, the average size (mean channel) had more than tripled, indicating a population of large, young platelets. Between the 11th and 16th days the average size dropped rapidly toward normal but then remained a little above normal until the 35th day, at which time the peripheral platelet count also returned to the control range. The size distribution of the red cell population remained essentially unchanged until the 21st day, when there was a shift from a single-peak curve to a markedly bimodal curve, indicating the appearance of a distinct second population with a larger average size. The simultaneous increase in the reticulocyte count to about ten times the control level indicated that the second peak of cell size corresponded to reticulocytes. As the reticulocyte number declined during the next ten days, there was a concurrent return to a unimodal curve of size distribution of erythrocytes, although the modal channel of the single peak was somewhat greater than that of controls for an additional week.

Measurements of the size distributions of platelet and erythrocyte populations indicate that at least 35 to 40 days are required for the kinetics of production of these cells to approach normal after exposure of the hosts to 950 r of whole-body radiation.

14.15 INFLUENCE OF MICROBIAL FLORA AND X RADIATION ON RETICULAR NEOPLASMS OF RFM MICE

H. E. Walburg, Jr. G. E. Cosgrove

The influence of microbial flora on radiation-induced myeloid leukemia has been reported previously.[1] This report deals with the influence of microbial flora on the development of thymic and nonthymic lymphomas, which are characteristically increased in incidence following x irradiation of RFM mice, and on reticulum-cell sarcoma, a spontaneous reticular neoplasm common in untreated mice of many strains, including the RFM. The relationship of myeloid leukemia to the other reticular neoplasms was also studied.

The following groups of male and female RFM mice, unirradiated or exposed to 300 r whole-body x rays at five to six weeks of age, were studied: (1) germfree (GF); (2) conventional (CONV); (3) conventionalized (CONVZ), which were transferred from an environment containing only bacteria of a limited number of genera to the conventional environment at time of irradiation.

The incidence of reticular neoplasms and mean age of death of these mice are shown in Table 14.15.1. X irradiation increased the incidence of thymic and nonthymic lymphoma in all three groups, to a greater extent in females than in males, with GF mice showing a greater incidence than either CONV or CONVZ mice. On the other hand, while irradiation increased the incidence of myeloid leukemia in CONV and CONVZ mice it failed to induce the disease in GF mice.

Irradiated mice in all groups had a lower incidence of reticulum-cell sarcoma than did their unirradiated counterparts. Furthermore, irradiated and unirradiated GF mice had a lower incidence of this disease than did their CONV and CONVZ counterparts, despite longer survival in the GF mice and hence greater numbers at risk to this late-occurring disease.

It is noteworthy that although GF mice had a higher incidence of radiation-induced lymphoma and a lower incidence of radiation-induced myeloid leukemia there was no difference in the combined incidence of these two diseases, suggesting that the microbial flora altered the type of radiation-induced reticular neoplasm but not the total incidence. On the other hand, the microflora did influence the incidence of reticulum-cell sarcoma. Further studies are under way to clarify the relationship of microbial flora to the pathogenesis of reticular neoplasms.

Reference

[1]H. E. Walburg, Jr., and G. E. Cosgrove, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967,* ORNL-4100, pp. 130–31.

Table 14.15.1. Incidence of Reticular Neoplasms and Mean Age at Death of Irradiated and Unirradiated RFM Germfree, Conventional, and Conventionalized Mice

X-Ray Dose (r)	Sex	Microbial Status	Number of Mice	All Causes (MAD ± SE)[a]	Myeloid Leukemia		Lymphoma		Reticulum Cell Sarcoma	
					Incidence (%)	MAD ± SE[a]	Incidence (%)	MAD ± SE[a]	Incidence (%)	MAD ± SE[a]
0	♀	GF	65	758 ± 25	0		12	680 ± 68	35	798 ± 31
		CONV	113	630 ± 15	0		7	594 ± 80	59	678 ± 13
	♂	GF	55	817 ± 16	0		4	830 ± 22	37	826 ± 27
		CONV	99	583 ± 16	1		5	574 ± 43	55	664 ± 12
300	♀	GF	98	417 ± 19	0		63	318 ± 15	5	517 ± 55
		CONV	91	350 ± 16	7	313 ± 64	58	284 ± 13	16	530 ± 40
		CONVZ	122	334 ± 11	7	352 ± 23	61	280 ± 10	18	431 ± 32
	♂	GF	82	583 ± 29	1		41	367 ± 38	21	696 ± 30
		CONV	99	476 ± 19	13	375 ± 34	27	305 ± 19	39	608 ± 17
		CONVZ	117	435 ± 16	20	408 ± 26	25	304 ± 23	24	574 ± 24

[a]Mean age at death ± standard error.

14.16 FOREIGN SPLEEN REACTION IN SUBLETHALLY X-IRRADIATED GERMFREE F_1 HYBRID MICE

H. E. Walburg, Jr. G. E. Cosgrove

The pathology of the graft-vs-host (GVH) reaction has been well characterized, but the role of secondary infection in the pathogenesis of this disease has not been resolved. Early experiments with germfree mice have resulted in further confusion because of a failure to quantitate the severity of the lesions. These experiments were carried out to help clarify the role of the microbial flora in the GVH reaction and to compare quantitatively the immunological reaction in germfree and conventional mice.

C3RF (C3H♀ × RFM♂) and RFC3 (RFM♀ × C3H♂) F_1 hybrid mice were exposed to sublethal amounts of x radiation and within 2 hr injected intraperitoneally with spleen cells from one of the parent strains. The mice were observed daily for death, and at various times after treatment, hemopoietic organs of the survivors were examined microscopically for characteristic lesions of the GVH reaction. The severity of the reaction was altered by varying the x-ray exposure and the number of parental spleen cells injected. In addition to x-ray exposure and number of parental spleen cells, recipient age influenced both survival and the development of histopathology. When recipient age was constant, the survival of mice receiving various lethal combinations of x-ray exposure and parental spleen cell dose was similar in germfree and conventional environments, and the reaction severity as measured by grading resultant lymph node granuloma was identical in germfree and conventional mice. When sublethal combinations of x-ray exposure and parental spleen cell dose were used, no difference in severity of the lesions in the lymph nodes of germfree and conventional mice was detected.

These results show that spleen cells from germfree parents are as capable of producing a GVH reaction in germfree irradiated F_1 hybrid recipients as are conventional cells in a conventional recipient and that the microbial flora plays little or no role in development of the pathology of the GVH reaction.

14.17 ^{125}I-LABELED IODODEOXYURIDINE INCORPORATION AS A MEASURE OF HEMOPOIETIC RECOVERY IN X-IRRADIATED MICE

H. E. Walburg, Jr. Edna I. Mynatt
E. A. Wulfsberg

Hemopoietic recovery following x irradiation has been studied most frequently by analysis of hemopoietic elements in the peripheral circulation and by use of radioactive-labeled metabolites to determine cell production. Common radioisotopes used are tritiated thymidine, which accurately depicts DNA synthesis but requires laborious techniques, and ^{59}Fe, which indirectly measures only one component of hemopoiesis, erythrocyte production. The thymidine analog ^{125}iododeoxyuridine (^{125}IUdR), which is a specific DNA precursor, can be used to assay cellular proliferation in vivo by scintillation counting of the ^{125}I gamma rays emitted from newly synthesized DNA. In this study, organ uptake of ^{125}IUdR was compared with ^{59}Fe incorporation, organ weight, organ cellularity, and appearance of mature hemopoietic elements in the peripheral circulation to assess hemopoietic recovery.

Hemopoietic recovery of the spleen, bone marrow, mesenteric lymph node, and thymus was studied at various times after exposure to 300 or 500 r of x rays in 12-week-old male or female CF No. 1 mice by measuring organ uptake of ^{125}IUdR. Bone marrow incorporation of ^{125}IUdR was severely depressed

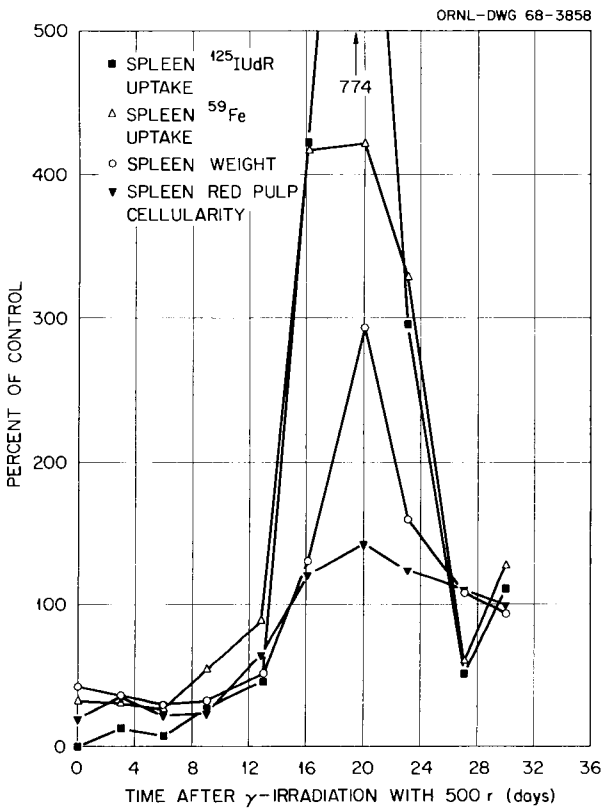

Fig. 14.17.1. Repopulation in the Spleen of Female CF No. 1 Mice After Whole-Body Exposure to 500 r x rays.

■——■ spleen ^{125}IUdR uptake
Δ——Δ spleen ^{59}Fe uptake
○——○ spleen weight
▼——▼ spleen red pump cellularity

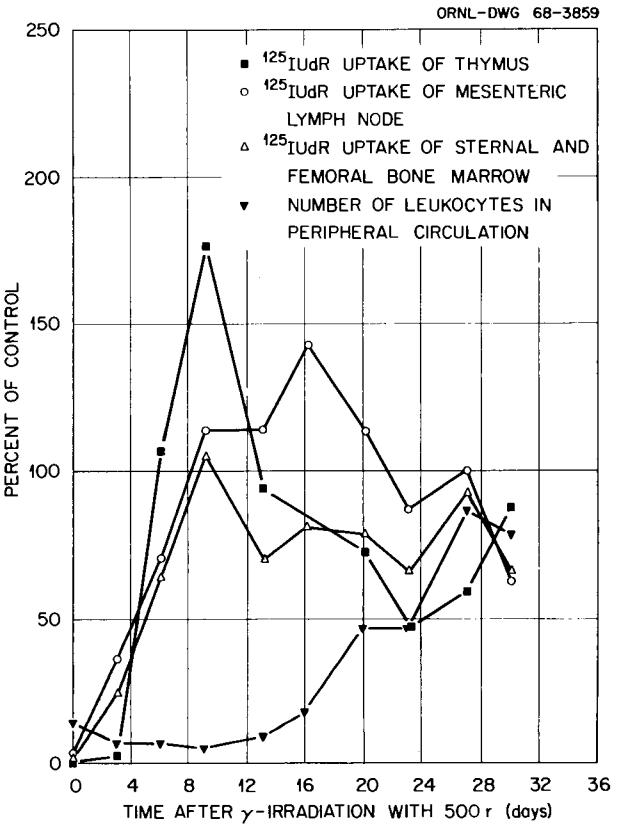

Fig. 14.17.2 Relationship of Repopulation in the Thymus to Repopulation of Other Tissues in Female CF No. 1 Mice Exposed to 500 r Whole-Body X Radiation.

■——■ ^{125}IUdR uptake of thymus
○——○ ^{125}IUdR uptake of mesenteric lymph node
Δ——Δ ^{125}IUdR uptake of sternal and femoral bone marrow
▼——▼ number of leukocytes in the peripheral circulation

immediately after x irradiation but then increased rapidly and reached the level of unirradiated controls by the end of the first week. In the spleen, however, ^{125}IUdR uptake was depressed for almost two weeks, with values higher than those of the unirradiated controls occurring in the third week (Fig. 14.17.1). Spleen ^{59}Fe incorporation, weight, and red pulp cellularity showed similar patterns of recovery. The thymus showed an early recovery similar to that seen in the bone marrow (Fig. 14.17.2), with ^{125}IUdR uptake rising above control values in the second week after irradiation but followed by a second depression occurring during the third and fourth weeks. Recovery in the mesenteric lymph node (Fig. 14.17.2) was similar to that in the thymus and bone marrow. Repopulation of the peripheral circulation with leukocytes did not begin until the second week, when thymus, bone marrow, and mesenteric lymph node activity were at a maximum.

These results are consistent with the thesis that organ incorporation of ^{125}IUdR is an accurate measure of hemopoietic recovery following x irradiation. Using this technique, the relationship of each of the hemopoietic organs to the others during repopulation after x irradiation is being studied.

14.18 CONCERNING THE SPECIFICITY OF TUMOR INHIBITION RESULTING FROM IMMUNIZATION WITH RAUSCHER LEUKEMIA VIRUS VACCINE

R. L. Tyndall J. A. Otten
N. K. Clapp

We have previously described a transformed growth pattern in an established BALB/c mouse cell culture (JLS V5) infected with the Rauscher leukemia virus (RLV). Such cells are highly tumorigenic when injected into newborn BALB/c mice and produce transplantable myxofibrosarcomas infected with leukemia virus. The corresponding uninfected control cells (JLS V6) are much less tumorigenic and on inoculation produce transplantable spindle-cell sarcomas free of infectious leukemia virus. Moreover, newborn mice of mothers immunized with RLV vaccine (formalized cell-free extract of leukemic spleen tissue from RLV-infected BALB/c mice) are resistant to challenge with transplantable myxofibrosarcoma cells, whereas control mice of mothers immunized with extracts of normal BALB/c spleen tissue are not resistant to tumor challenge. The present study

Table 14.18.1. Specificity of the Tumor Inhibition Resulting from Immunization with Rauscher Leukemia Virus Vaccine

Tumorigenic Challenge (Cells or Chemical)[a]	Vaccine	No. Tumor-Positive Animals/ No. Injected	Percent	Tumor Type
Myxofibrosarcoma	Rauscher leukemia virus	0/54	0	
	Control	45/54	83	Myxofibrosarcoma
Spindle cell sarcoma	Rauscher leukemia virus	7/7	100	Spindle cell sarcoma
	Control	28/28	100	Spindle cell sarcoma
Spindle cell sarcoma plus irradiated myxofibrosarcoma	Rauscher leukemia virus	23/23	100	Spindle cell sarcoma
	Control	8/8	100	Spindle cell sarcoma
Diethylnitrosamine	Rauscher leukemia virus	18/23[b]	87	Multiple types[c]
	Control	20/23[b]	78	Multiple types[c]

[a]DEN given orally in drinking water.

[b]Number of tumor-bearing animals/number of survivors histologically examined.

[c]For further details see companion report in this publication.

was undertaken to analyze further the specificity of tumor inhibition by immunization with RLV vaccine.

In one series of experiments, groups of newborn mice of mothers immunized with either RLV vaccine or control vaccine (vaccine prepared from normal BALB/c spleen tissue) were challenged with either transplantable spindle-cell sarcoma (originating from uninfected JLS V6 cells) or myxofibrosarcoma cells (originating from RLV-infected JLS V5 cells). Another group of mice passively immunized with either RLV or control vaccine and challenged with spindle-cell sarcomas were also concurrently challenged with irradiated (10,000 r) myxofibrosarcoma cells as a source of leukemia virus, to determine if concurrent infection with virus would alter their response to tumor challenge. In another series of experiments adult BALB/c mice immunized with either RLV vaccine or control vaccine were then given diethylnitrosamine (DEN) in the drinking water daily for 21 weeks (survivors received an average cumulative dose of 871 mg of DEN per kilogram body weight). As seen in Table 14.18.1, immunization of mice with RLV vaccine afforded complete protection against myxofibrosarcoma challenge but afforded no protection against spindle-cell sarcomas or tumors induced by DEN.

The inability of immunization with RLV vaccine to protect mice against tumor induction by DEN and, in particular, against challenge with spindle-cell sarcomas, with or without added leukemia virus, indicates specificity in the protective effect of such immunization against the myxofibrosarcoma. This suggests the presence of a hitherto unrecognized transplantation antigen common to both leukemic spleen cells and the myxofibrosarcoma cells. The induction of this antigenic specificity may be indicative of an additional property applicable in the classification of the murine leukemia viruses. Studies are presently under way to determine if this antigen is RLV-induced protein or is RLV protein per se.

14.19 FURTHER PURIFICATION OF MOUSE PLASMA ERYTHROPOIETIN AND THE EFFECT OF ITS PRESENCE ON PLASMA ESTERASE ISOENZYMES

R. C. Allen D. J. Moore
J. W. Fisher[1]

High-resolution analytical and preparative flat-bed discontinuous electrophoresis in acrylamide gel has demonstrated that erythropoietin is associated with the band 6 cholinesterase isoenzyme in mice made anemic by phenylhydrazine treatment. Further, the relative specific activity of this isoenzyme is markedly decreased as erythropoietin levels increase in both phenylhydrazine-induced anemia and in mice made hypoxic in silicone membrane chambers. Upon cessation of either treatment the relative activity returns to normal levels. This might suggest that the band 6 cholinesterase is simply acting as a carrier with erythropoietin saturating the active esterase sites. The present efforts to purify band 6 were aimed at exploring this possibility.

As demonstrated by schlieren optical patterns of primary flat-bed discontinuous electrophoretic separations of mouse plasma, band 6 has been separated into six individual components, three of which have esterase activity. Biological assay of these individual proteins has shown that two bands, tentatively designated as 6c and 6*d*, have erythropoietin activity. Band 6*d* is an esterase, while band 6c is not.

Thus, there appear to be two distinct electrophoretic species of erythropoietin present in the plasma of mice made anemic by phenylhydrazine treatment. To ascertain whether bands 6c and 6*d* are carriers, these bands are being separated from both phenylhydrazine-treated and normal mice and examined further. In addition, the roles of the band 7 and band 12 esterase isoenzymes, which also show marked changes in relative activity during erythropoietin production in the mouse, are being analyzed similarly.

Reference

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14.20 ERYTHROPOIETIN INHIBITOR IN KIDNEY EXTRACTS AND PLASMA FROM ANEMIC UREMIC HUMAN SUBJECTS

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B. L. Roh[1] R. C. Allen
B. J. Kelley[1]

The pathogenesis of the erythroid failure in the anemia of chronic renal insufficiency has been postulated to be due to diminished erythropoietin

(ESF) production and/or the toxic effects of the metabolic products of uremia. We have previously demonstrated an ESF inhibitor in rat kidney homogenates. In the present studies the concentrations of ESF inhibitor in the soluble cytoplasmic fractions of kidneys from two patients with chronic glomerulonephritis and two normal humans were compared.

Inhibitor levels in nephritic kidneys (amount necessary to produce 50% inhibition of 0.2 unit ESF determined by ^{59}Fe incorporation in RBC of polycythemic mice) were approximately 16 times greater than that in normal kidneys. Uremic plasma completely blocked ESF (1 ml plasma per 0.2 unit per mouse), whereas normal plasma accentuated ESF. Daily injections (seven days) of nephritic kidney homogenates into normal mice produced decreases in hematocrit and ^{59}Fe incorporation by RBC. Protein pherograms (flatbed discontinuous electrophoresis) on the nephritic kidney soluble fractions, when compared with those from normal kidney fractions, revealed a marked increase in the albumin region, the appearance of an additional band in the region adjacent to the albumin fraction, a decrease in an esterase positive protein (band 11), and a decrease in an α-1-globulin that migrated similarly to plasma ESF.

These preliminary data indicate that the kidneys and plasma from patients with anemia or uremia have elevated levels of an ESF inhibitor and support the hypothesis that anemia of chronic renal insufficiency is probably due to an increase in ESF inhibitor and a decrease in available ESF.

Reference

[1]University of Tennessee Medical Units, Memphis (supported in part by USPHS grants AM-02973 and AM-09027).

14.21 POSSIBLE USE OF ENZYME MARKERS TO FOLLOW THE GENETIC STABILITY OF DONOR AND HOST CELLS IN MOUSE RADIATION CHIMERAS

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An esterase enzyme marker (Es 2b) present in sublines of a new mouse strain, RF/Al$^+$, and absent in another, RF/Al$^-$, has been shown to be suitable for use as a genetic tracer.

Injection of lethally x-irradiated RF/Al$^-$ mice with RF/Al$^+$ spleen cells resulted in consistent appearance of the donor-specific esterase in the spleen and nodes of the recipients. This indicates genetic stability of donor hematopoietic cells, in conformity with the results of other studies. When chimeric plasmas and livers were studied, a few also showed Es 2b to be present. On the basis of our previous work suggesting that circulating cells may transfer nuclear material on the chromosomal level to rapidly replicating parenchymal cells, the explanation of these results we favor is that new genetic information was added to small numbers of host cells which could then direct the formation of the specific esterase enzyme.

In addition to providing a new test for chimerism, the Es 2b system has demonstrated several advantages of enzyme marker systems. Such systems can be applied to a variety of cellular and humoral materials. Where the enzyme is not membrane-related, it can be used to supplement antigenic markers. Furthermore, enzyme markers can be quantitated accurately and reproducibly with densitometers. As sensitive reflections of genetic activity, such markers may be used to study whether or not genetic material can be added and deleted from cells in vivo. It is hoped that the development of this and other cell- and strain-specific phenotypic markers will accelerate progress in immunogenetic research.

Reference

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15. Cell Growth and Differentiation

Tuneo Yamada	
Control of Cell Differentiation (15.1–15.11) Tuneo Yamada D. S. McDevitt[a] S. P. Modak[b] Egidio Puccia[b] Rolf Nöthiger[b] A. F. Van Dam[b, c] Lola M. Kyte D. H. Reese Marion E. Roesel Isaura Meza[b]	**Chromosome Ultrastructure (15.12, 15.13)** O. L. Miller, Jr. George Gassner III[c] Barbara R. Beatty **Vitellogenesis (15.14–15.19)** R. A. Wallace J. N. Dumont Marilyn S. Kerr[c] D. W. Jared Diana M. Rudack[d]
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15.1 SEQUENTIAL CHANGES IN THE SEDIMENTATION PROFILE OF RNA SYNTHESIZED DURING TISSUE TRANSFORMATION

D. H. Reese Egidio Puccia
Tuneo Yamada

In an effort to study the mechanism involved in transformation of newt iris into lens in Wolffian lens regeneration, we have accumulated autoradiographic data related to RNA synthesis.[1,2] These data are admittedly insufficient for assessing the role of transcription in the tissue transformation. A biochemical approach to the problem has been badly needed. However, the minute quantity of tissue available has resisted application of conventional biochemical methods. In the present study the difficulty is overcome to a certain extent by labeling tissue in vitro and applying the sucrose density gradient method to a mixture of labeled regenerate RNA and nonlabeled carrier RNA. Information concerning the type of RNA synthesized at various stages of tissue transformation is beginning to be accumulated. Evaluation of the data together with autoradiographic data enables us to make some conclusions on the pattern of RNA synthesis characteristic to each developmental phase of regenerate cells.

The lens regenerates of adult *Triturus viridescens* at various stages were surgically isolated in a slightly modified Wolff-Quimby amphibian culture medium adjusted to pH 7.4, to which 2 μc/ml of ^{14}C (U)-uridine was added. Culturing was performed on a rocker platform at 25°C for 2 or 5 hr in a CO_2 incubator. In the series of cold phenol extraction, the following procedures were used: After termination of culture, the regenerates were thoroughly washed and slowly homogenized in 0.01 *M* Tris-HCl buffer pH 7.4 containing 0.14 *M* NaCl, 0.01 *M* KCl, 0.0015 *M* $MgCl_2$, 1% SDS, and 200 μg PVS/ml. The homogenate was extracted with 80% aqueous phenol at 4°C after-added with the carrier

RNA from rat liver. After centrifugation, the aqueous layer was removed and $^1/_{10}$ final volume of 20% potassium acetate pH 5.2 was added, and the RNA was precipitated with 2 volume of 95% ethanol. In the series of hot phenol extraction, the homogenization medium consisted of 0.02 *M* potassium acetate, 0.28 *M* LiCl, 1% SVS, and 200 μg PYS/ml. After addition of carrier RNA, the homogenate was shaken at 60°C for 10 min with an equal volume of phenol. RNA samples were layered over 5 to 20 or 10 to 40% sucrose density gradient at pH 7.35.

In the main series of experiments reported here, 5 hr labeling was followed by cold phenol extraction. The primary purpose of the series was to obtain information on rRNA synthesis. For preliminary identification of rRNA's, 28 S and 18 S peaks of carrier RNA were used as measures. Since those two S values are attained by newly synthesized rRNA molecules after a process of conversion, it was pertinent in these experiments to allow a sufficient time for the conversion to proceed. On the basis of autoradiographic data[2] on the time needed for spreading of RNA radioactivity from the nucleus to the cytoplasm in newt lens regenerate cells, 5 hr incubation at 25°C appeared to be adequate. The proposal was justified by the observation that the cell population which gave rise to distinct 28 S and 18 S peaks of radioactivity after 5 hr of incubation failed to show such peaks when incubated only for 2 hr.

The sedimentation profile of radioactivity after 5 hr labeling and cold phenol extraction showed the following changes in the course of tissue transformation. In the normal dorsal iris which furnishes the cell material for the new lens, incorporation of the labeled precursor into 28 S and 18 S regions is insignificant. In the four-day dorsal iris, in which cells are still heavily pigmented but are already activated to replication, the incorporation into two peaks becomes definite. The most prominent incorporation into those two areas occurs in the 15-day regenerates, in which a large number of cells are depigmented, arranged in the lens vesicle, and mostly in the phase of active proliferation. In the 23- and 62-day regenerates, in which morphological specificity of lens tissue has been established and the lens epithelium containing replicating cells and the fiber area containing nonreplicating cells have been segregated, 28 S and 18 S areas show prominent peaks of radioactivity. The fact that the removal of the lens epithelium from the regenerating lens before incubation almost completely suppresses incorporation into these two peaks indicates that lens epithelial cells are responsible for synthesis of rRNA at these stages.

Besides the above-discussed peaks at 28 S, 18 S, and 4 to 5 S areas, a number of smaller peaks can be distinguished in the radioactivity curve of the series after cold phenol extraction. Some of them appear to be stage specific. For instance, the 15-day or later series indicate a peak around the 25 S area, which is lacking in the four-day as well as zero-day series. The difference in sedimentation profiles of radioactivity between 4- and 15-day series is pronounced when extraction was done with hot phenol. Interpretation of these observations needs further studies.

The peak in the 4 to 5 S region is present in all series described above. It increases strongly from the 0-day to 4-day series and attains the largest amount at 15-day series and slightly declines later. Even the lens without epithelium shows incorporation in this area. We do not have data to decide how much of the incorporation is due to terminal addition to tRNA. An answer is expected from a study of actinomycin effects on incorporation now being conducted.

The obtained data together with available background information[3,4] indicate that lens removal strongly enhances rRNA synthesis in the iris epithelial cells within four days after lens removal. A high level of rRNA synthesis is maintained by the cells in the depigmentation and multiplication phases, but is terminated when the cells enter the fiber differentiation phase. In other words, enhanced rRNA synthesis temporally coincides with cell replication activated in this system and also with the sensitivity toward actinomycin treatment.[5] The data reported also suggest that another type of sequential changes in the RNA profile occurs as the cells pass from the phase of dedifferentiation to the phase of metaplastic redifferentiation. The nature of such changes will be the object of further studies.

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15.2 CULTURE OF IRIS CELLS FROM *TRITURUS VIRIDESCENS*

D. H. Reese

The first conspicuous morphological event in Wolffian lens regeneration is a depigmentation of the dorsal pupillary iris margin. Previous in vitro studies on Wolffian lens regeneration have shown that this event does not take place when the iris is put into tissue culture[1,2] even if it is cultured in the presence of neural retina,[3] which is the hypothesized "inducer" of lens regeneration. However, depigmentation and lens regeneration will take place in vitro if the iris is maintained within the ocular environment.[2] There are obviously some subtle conditions necessary for the initiation of lens regeneration, which till now have not been reproduced in vitro outside the ocular environment. We have now found that depigmentation can be induced in the normal iris by trypsinization of the iris and subsequent culture of the dissociated cells.

Using sterile procedures throughout, the dorsal half of 29 irises was removed from the adult newt *Triturus viridescens* and dissociated at 25°C in 5 ml 0.25% trypsin solution made up in half-strength calcium- and magnesium-free phosphate-buffered saline (Dulbecco). Cells were harvested at 15-min intervals and centrifuged at 300 × *g* for 5 min. The cells were then resuspended in 4 ml amphibian adjusted Leibovitz L-15 culture medium with 10% fetal calf serum and cultured at 23 to 24°C in small plastic culture flasks. While all four harvestings produced enough cells for starting cultures, the second harvesting produced the best culture. The others were maintained for a time and then discarded.

Cells attached quickly and began to depigment in a way which is characteristic of in vivo lens regeneration but different from that of other melanin-containing cells in culture so far studied. Melanin-containing cells from other tissues lose their melanin when put into culture by a dilution process due to cell division.[4] The newt iris cells, however, actively expel melanin; for a period of one to two weeks after setting up the cultures, free melanin granules were evident in the cultures and were poured off several times a week. These cells, now completely depigmented with the exception of a very small number which have either retained or have synthesized new melanin, have been kept in culture for four months and have been subcultured seven times. Cultures have been successfully divided up to 1 : 20 but are normally divided 1 : 5. Although no data are available yet on the kinetics of mitosis of these cells, the number of mitotic figures is very high in new subcultures but decreases drastically when a monolayer condition is approached. It should be added that the normal iris of the adult newt is nondividing. Thus it is evident that activation of cell replication and depigmentation which characterize Wolffian lens regeneration can be induced in the normal iris of adult newts by putting the tissue in cell culture. Experiments are planned to see if cloning of pigmented iris cells leads to the same result, and if these cells can be influenced by neural retina to synthesize lens proteins in vitro.

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15.3 LOCALIZATION OF LABELED ACTINOMYCIN D IN DIFFERENTIATING LENS REGENERATE CELLS

Tuneo Yamada Marion E. Roesel

In our earlier study[1] concerning the effect of actinomycin D on morphogenesis in the newt lens regenerating system, the cells in the multiplication phase were strongly affected and inhibited in development, while the cells in the early part of fiber differentiation phase were not affected and proceeded in development. One of the questions raised was whether this differential effect was due to difference in penetration of the antibiotic into

cells in different phases of differentiation or due to difference in inherent properties of cells in various phases. To approach this problem, an autoradiographic study of the distribution of tritiated actinomycin D in lens regenerate cells in various phases was carried out.

The lens regenerates of stages VI to IX of adult *Triturus viridescens* were surgically isolated, cultured in a modified Wolff-Quimby medium containing 0.5 μc/ml of ^{3}H-actinomycin D (3.8 curies/millimole) for 2 hr at 25°C in a CO_2 incubator, and studied in autoradiographic sections.

In all types of regenerate cells, silver grains are preferentially localized over nuclei. When the average grain counts per cell of different cell types are compared, the cells in active depigmentation (18.62), immediately after depigmentation (18.18), in the lens epithelium (19.42), and in the early phase of fiber differentiation (20.44) show similar values. This implies that the differential effects of actinomycin on development of replicating depigmented cells and that of cells in the early phase of fiber differentiation reported earlier[1] are not due to differential penetration of actinomycin into nuclei of these cell types. On the other hand, the cells in the middle phase of fiber differentiation have very low average counts (6.26). In the regenerating lens the last type of cells are located in the interior of a tissue complex 100 to 300 μ in diameter. Thus it appeared possible that low counts of those cells are due to slow diffusion of actinomycin through the overlying tissue. However, observations of fragmented regenerate lens show that the nuclei of such cells take up significantly less radioactivity even when they are situated close to the tissue surface. This seems to suggest that the ability of nuclei to bind actinomycin is reduced during fiber differentiation. That extensive ultrastructural changes occur in the nuclei during this phase has been already reported.[2] In the above discussion it is assumed that the present technique detects actinomycin bound to cell structures.

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15.4 AVAILABILITY OF INTRAPERITONEALLY INJECTED TRITIATED THYMIDINE FOR LABELING LENS REGENERATE CELLS TRANSPLANTED IN THE NEWT OPTIC CHAMBER

Tuneo Yamada Marion E. Roesel

Injection of tritiated thymidine has been extensively used in the newt for studying cell replication, which plays a decisive role in transformation of the iris into lens. These studies are based on the assumption that the time interval during which the injected labeled precursor is available for labeling cells in the adult newt is relatively short. The assumption appeared justified by the data on the radioactivity of the serum and on the average grain counts of labeled cells subsequent to injection of the labeled precursor. Those data show the availability time to be from 1 to 3 hr. However, some papers suggest an extremely long availability time of injected tritiated thymidine, extending to more than one day in the adult newt and frog. The present work is designed to determine the time interval during which the DNA-synthesizing cells in the newt optic chamber are labeled after intraperitoneal injection of tritiated thymidine.

The method consists in grafting nonlabeled lens regenerate tissue of adult *Triturus viridescens* into the optic chamber of another animal 0 to 24 hr after intraperitoneal injection of 1 μc of tritiated thymidine per gram of body weight, and in studying labeling of cells in the proliferating zone of the grafted lens regenerate by autoradiography. The average grain count per labeled cell and the labeling frequency of cells in the proliferating zone of the lens regenerate, which was grafted after different time intervals after injection, were studied. This method gives information on availability of extracellular labeled precursor at a given time, but not on that of labeled precursor present in the intracellular pool.

In the 0- and 1-hr series all grafts indicated labeled cells. The average grain counts of labeled cells decreased sharply from 0 to 1 hr. In the 3-hr series two-thirds of cases showed no labeled cells, while one-third showed labeled cells with very low average grain counts. No labeled cells were detected in all grafts studied in the 4-hr and subsequent series. We conclude that tritiated thymidine remains available for cellular DNA synthesis in the optic chamber until *ca.* 3 hr and becomes un-

available before 4 hr from the time of intraperitoneal injection under our experimental conditions.

15.5 HISTOLOGICAL AND CYTOLOGICAL PATTERNS OF INCORPORATION OF TRITIATED URIDINE INTO LENS REGENERATES CULTURED IN VITRO

Tuneo Yamada Marion E. Roesel

In application of the sucrose density gradient method to a study of the sedimentation profile of RNA synthesized at various stages of Wolffian lens regeneration,[1] two technical questions were raised which had to be answered before making any serious attempt along this line. One of the questions was whether or not the culture technique used for labeling regenerate RNA with ^{14}C-uridine affects the in vivo pattern of incorporation of the precursor into RNA. Since application of biochemical methods to RNA of lens regenerates labeled in vivo is not technically feasible, the only way to check this point is to use autoradiography. The second question is concerned with the iris stroma, which does not participate in lens regeneration but had to be included in the culture of lens regenerates during the labeling period in 0-, 4-, and 15-day series. We have to know to what extent the stroma contributes to radioactivity in RNA in the total tissue under our experimental condition. The present experiments are designed to answer these questions with autoradiography.

Four- and fifteen-day lens regenerates with accompanying stroma were isolated from adult *Triturus viridescens* according to the technique used in the above-cited experiment[1] and cultured in the same medium. After a 1-hr stay in the medium containing 2.5 μc/ml of ^{3}H-uridine, a portion of the explants were fixed, and the rest of the explants were transferred, after washing, into the same culture medium without the labeled precursor, cultured for 5 hr, and fixed. Other culture conditions were the same as in the cited experiments. The tissues were fixed in acetic acid—ethanol mixture and embedded in paraffin or methacrylate. Paraffin sections 5 μ thick or methacrylate sections 0.5 μ thick were covered with liquid emulsion NTB 3 (Kodak).

The distribution of silver grains over the nucleus and cytoplasm in the series of 1-hr pulse and that of 1-hr pulse followed by 5-hr chase in the 4- and 15-day series was studied and compared with the corresponding series of autoradiographs of lens regenerates labeled in vivo.[2,3] No difference could be detected in the distribution pattern of silver grains within the cell and its change in time between in vitro and in vivo series. However, in the in vitro series a small fraction (less than 5%) of iris epithelial cells indicate no silver grains at all. These negative cells often contain necrotic nuclei and are interpreted as cells damaged by surgical operation.

By counting silver grains over the lens regenerate proper and those over the iris stroma including blood cells, an estimate of radioactivity in stroma compared with total radioactivity was made. The data indicate that the percentage of stroma grains in the total grains was 1.6 for the 4-day series and 0.9 for the 15-day series. Calculation shows that in the 0-day series the same percentage should be 5 to 7%. It is concluded that the radioactivity in the stroma does not significantly affect the sedimentation profile of the 4- and 15-day series but may affect that of the 0-day series. All data obtained in this report have been considered in interpretation of results in the above-cited experiments.[1]

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15.6 IMMUNOFLUORESCENCE STUDIES ON THE LENS-SPECIFIC PROTEINS IN AMPHIBIAN LENS DEVELOPMENT

D. S. McDevitt Isaura Meza

Various physicochemical criteria have enabled workers to recognize today in the vertebrate lens three groups of tissue-specific proteins: the classical α, β, and γ crystallins. The synthesis of γ crystallins, that group of lens proteins lowest in molecular weight and electrophoretic mobility, has been implicated by several groups of workers as the primary event in vertebrate lens fiber differentiation.[1,2] In the present investigation, the lens-specific proteins, especially the γ crystallins,

were examined in the amphibian *Rana pipiens* during the lens induction and differentiation stages. The lens proteins of this organism, separated initially by ion exchange chromatography,[3] were characterized immunologically and utilized as antigens in an immunofluorescence investigation of their ontogeny and localization in lens development.

The supernatant of a whole-lens homogenate obtained from adult *R. pipiens* lenses was fractionated on a DEAE-cellulose column. The γ crystallin fraction so produced was further purified by gel filtration on a Sephadex G-100 column. The antigens to be employed, total lens protein and the isolated γ crystallins, were tested for immunological specificity using immunodiffusion and immunoelectrophoresis techniques.

The crude globulin fraction of several antisera to these antigens was conjugated with the fluorescent dye fluorescein isothiocyanate ("direct" technique), or used in conjunction with a goat antirabbit γ globulin ("indirect" or "sandwich" technique) to detect the presence and localization of the homologous antigen in the lenses of embryonic *R. pipiens*. Such specific reagents were applied to 3-μ paraffin sections through the head region of Shumway stages 16 (neural tube) to 25 (operculum complete), which had been fixed in either cold Carnoy's fixative or cold 95% ethanol and further processed at 5°C. The substitution of conjugated or unconjugated nonimmune rabbit γ globulin for the specific antibody was used as a control.

The initial γ crystallin antigen isolated by DEAE-cellulose chromatography cross-reacted with other lens protein fractions when tested immunoelectrophoretically against an anti-total lens protein antibody. Because of this indication of incomplete separation of the crystallins, the above γ crystallin fraction was rechromatographed on Sephadex G-100, resulting in a more purified γ crystallin fraction. Used as an antigen, it produced a specific anti-γ crystallin antibody suitable for use as a *specific* marker in the immunofluorescence detection of γ crystallins.

The results obtained indicate that γ crystallins are first discernible at Shumway stage 20 in a few prospective primary lens fiber cells, which are located in the internal layer of the lens vesicle. The fluorescence due to the presence of γ crystallins in the fiber area increases until stage 25. The external layer of the lens vesicle and the lens epithelium appear to lack any specific staining for γ crystallins throughout this period. At stage 25, the lens epithelium begins to exhibit a weakly positive immunofluorescence for γ crystallins, while the cells in the fiber area exhibit the same immunofluorescence very strongly.

When sections of embryonic lenses are treated with an anti-total lens protein antibody, fluorescence is first visible at stage 20, as with the anti-γ crystallin antibody. However, in the immediately following developmental stages, the external layer of the lens vesicle and, later, the lens epithelium exhibit the immunofluorescence reaction as well as the fiber area. It should be noted that at no time did application of either the anti-total lens protein or anti-γ crystallin antibodies result in fluorescence in any eye tissue prior to stage 20, the lens vesicle stage.

Thus in this organism the appearance of γ crystallins is indicative of lens fiber differentiation, as has been reported in the regenerating urodelan lens[1] and bovine mammalian lens.[2] Moreover, these results lend credence to the concept that the lens-specific proteins are synthesized after the inductive interaction of the optic cup and overlying ectoderm has occurred, that is, they are *not* necessary prerequisites for the establishment of lens rudiment and lens morphology, as earlier suggested by other workers.

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15.7 IMMUNO-ELECTRON MICROSCOPIC STUDIES ON THE LOCALIZATION OF γ CRYSTALLINS IN AMPHIBIAN LENS CELLS

Rolf Nöthiger D. S. McDevitt
Isaura Meza

When a lens is formed, either during regeneration or embryonic development, its cells undergo a sequence of well-defined changes. The final event is the production of lens fibers and their main pro-

teins, the crystallins, notably γ crystallins.[1] Attempts are being made to study the intracellular localization of these proteins. We are reporting here our progress made in developing an immunoelectron microscopic technique.

Nakane and Pierce[2] recently reported on the usefulness of horseradish peroxidase as a label for antibodies. This enzyme offers certain advantages over the "classical" marker, ferritin: its smaller molecular weight (44,000 as compared with *ca.* 700,000) should allow better penetration into the cells; and its reaction product is visible in the light microscope as well as in the electron microscope.

Among several fixatives tried, 4% buffered formaldehyde best preserved antigenic specificity as well as ultrastructural details, as judged by immunofluorescence and electron microscopy. Lenses of *Rana pipiens* embryos, fixed in formaldehyde and embedded in paraffin or Epon, served as a test system. The indirect technique was used, with antibodies specific against *R. pipiens* γ crystallins as the intermediate layer.

Peroxidase was conjugated to goat anti-rabbit globulins with *p,p'*-difluoro-*m,m'*-dinitrodiphenyl sulfone as a coupling agent. The bulk of unreacted free peroxidase was removed from the crude conjugate in 50% saturated $(NH_4)_2SO_4$. The precipitate was redissolved in phosphate-buffered saline and passed through a Bio-Gel P-300 column. We were able to separate satisfactorily the conjugated antibodies from the other components of the reaction mixture. Four peaks were obtained representing in the order of decreasing molecular weight: "denatured" proteins, conjugated globulins, unconjugated globulins, and remaining free peroxidase respectively. Agar-immunodiffusion tests revealed cross reaction with rabbit gamma globulins as the antigen in the second and third peak. If the same fractions were applied to paraffin sections of *R. pipiens* lenses, specific staining was found among the fractions of the second peak only. These fractions were subsequently pooled, absorbed three times with mouse tissue powder, dialyzed vs H_2O, and lyophilized. Specific staining occurred only in cells in the fiber differentiating phase, but not in the lens epithelial cells. These results confirm the findings of McDevitt and Meza,[3] who used fluorescent antibodies and the direct technique.

As was expected, the penetration of the antibodies into the cells proved to be a very difficult technical problem. So far the problem has not been solved. Fragments of *R. pipiens* lenses which had been fixed, frozen, and thawed were floated in antibody solution without evidence of penetration.

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15.8 PREPARATION OF SPECIFIC ANTIBODIES AGAINST γ CRYSTALLINS OF THE NEWT *TRITURUS VIRIDESCENS*

D. S. McDevitt Rolf Nöthiger

Takata *et al.*[1] applied fluorescent antibodies which were specific for *Triturus* lens γ crystallins to regenerating lenses of *Triturus viridescens*. They detected γ crystallins in the cytoplasm as well as in the nuclei of differentiating fiber cells. In contrast to this, no immunofluorescence was observed in nuclei of *Rana pipiens* embryonic lenses with *R. pipiens* anti-γ crystallin or anti-whole lens protein antibodies (McDevitt and Meza).[2] These antibodies also fail to stain nuclei of young *Triturus* regenerates. Only in advanced primary fiber cells could we detect some nuclei with specific staining. The difference between Takata's and our results is not easily understood, nor was it expected, since anti-γ crystallin of *R. pipiens* cross-reacts with *Triturus* whole lens proteins and γ crystallins (agar immunodiffusion). It therefore became desirable to obtain specific antibodies against *Triturus* γ crystallins in order to compare their reaction with that of *R. pipiens* anti-γ crystallins.

Five hundred lenses of *Triturus viridescens* were homogenized in 0.005 *M* phosphate buffer, and from this a total of 218 mg of soluble proteins were obtained. Two hundred milligrams in 9.2 ml of buffer were fractionated on a DEAE-cellulose column using an NaH_2PO_4-Na_2HPO_4-NaCl buffer system, pH 7.0 to 5.7. Details of the method are given by McDevitt.[3] The chromatographic pattern obtained is shown in Fig. 15.8.1. For comparison (dashed line) a proportionally reduced profile of *R. pipiens* lenses is shown so that the two γ crystallin peaks coincide. Compared with *R. pipiens*

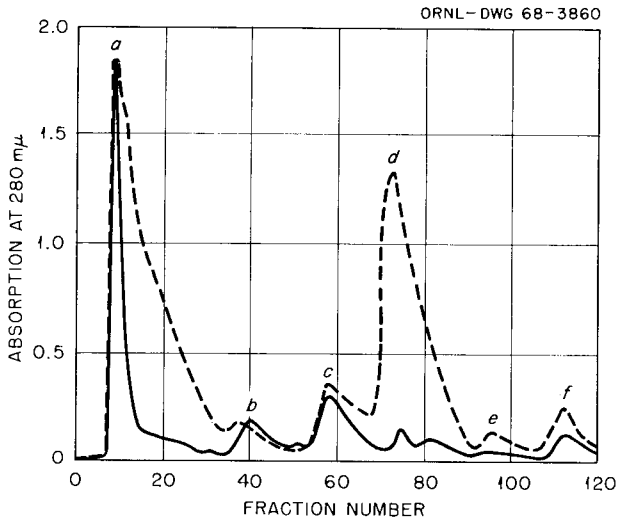

Fig. 15.8.1. Chromatographic Pattern of Adult *Triturus viridescens* (Solid Line) and *Rana pipiens* (Dashed Line) Whole Lens Proteins, Fractionated on 10 g of DEAE-Cellulose. The elution pattern for *Rana pipiens* has been proportionally reduced so that the two gamma-crystallin peaks coincide. Ten-milliliter aliquots were collected. (*a*) Gamma crystallins, (*b* and *c*) beta crystallins, (*d*, *e*, and *f*) alpha crystallins.

lens, *Triturus* lens contains relatively more γ crystallins, which are considered to be the primitive lens proteins. In this respect *Triturus* is phylogenetically closer to the teleosts, which have γ crystallins as their predominant lens protein. Birds, on the other hand, have little or no γ crystallin (Maisel and Goodman).[4]

The γ crystallins in fractions 8 to 25 were precipitated with 80% saturated $(NH_4)_2SO_4$, redissolved in 0.05 *M* Tris-HCl buffer, pH 7.2, centrifuged, and passed through a Sephadex G-100 column for further purification of these fractions. The γ crystallins with a molecular weight of *ca.* 20,000 were eluted last. Eleven fractions from this peak with a total volume of 33 ml were dialyzed vs H_2O and lyophilized. About 15 mg of purified γ crystallins were harvested.

For the production of specific antibodies, a rabbit was injected subcutaneously with 4 to 5 mg of γ crystallins in complete Freund's adjuvant (1 : 1). A second identical injection was given three weeks later. Eighteen days after the second injection 1.5 ml of blood was taken and the serum used for immunological tests. In agar-immunodiffusion analyses 10 μl of serum could detect 0.1 μg of the homologous antigen. Further tests to confirm the purity and specificity of this antibody are in progress.

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15.9 STUDIES ON TERMINAL CELL DIFFERENTIATION IN CHICK LENS. I. ANALYSIS OF NUCLEAR DEGENERATION, PLOIDY, AND DNA LOSS

S. P. Modak Stella W. Perdue[1]
R. F. Kimball[1]

It has been known for a long time that during the later part of development of chick lens, cell nuclei degenerate and finally disappear from the lens fiber area. However, no detailed study is yet available pertaining to the temporal sequence of nuclear degeneration and the consequent changes in the DNA content. Since the loss of nuclear structure and function should be the consequence of entrance of cells in the last phase of cell differentiation, such a study is warranted.

Chick embryonic lenses of 4 to 20 days of development and post-embryonic lenses of 0 to 10 days of age were fixed in Carnoy (6 : 3 : 1) and serially sectioned at 5 μ thickness. At least four lenses of each stage were treated in this manner. Sections were stained in Mayer's hemalum and mounted subsequently in Canada balsam. A few sections were also stained by Feulgen reaction.

Chick lenses of various ages (4, 6, 13, and 16 days embryonic stage and 0 and 10 days post-embryonic age) were dissected out in cold Tyrode solution. From each lens, lens epithelium and lens fiber area were microsurgically isolated. In the older lenses an area intermediate to the lens epithelium and the lens fibers was similarly isolated. The isolates were squashed on separate slides and placed on Dry Ice to remove the cover

slip. Preparations were fixed in cold Carnoy. After the removal of the fixative, preparations were subjected to hydrolysis in 1 *N* HCl for 8 min at 60°C and subsequently stained with Schiff's reagent for 60 min, followed by treatment in metabisulfite baths. Stained preparations were partially dehydrated and then air-dried. In some cases, smears of adult chicken sperm were applied adjacent to the lens epithelium squashes before the fixation of the latter and then treated as above.

Slides were mounted in refractive index oil in order to reduce loss of light by nonspecific scattering. The amounts of Feulgen-positive material in individual intact nuclei were measured by the single-wavelength method on the Zeiss USMP I microspectrophotometer at 559 mμ.

It was found that first indications of nuclear degeneration become apparent at about nine days of embryogenesis. This is indicated by the appearance of nuclei with condensed chromatin in the center of the lens fiber area. During the earlier phase of pycnosis, nuclei show very heterogeneous staining characteristics. Later on, the chromatin clumps into a rounded, dense, heavily staining structure. Progressively, the dense pycnotic nucleus decreases in size and in staining intensity, finally disappearing completely. At about 13 days of development, massive pycnotic changes are visible in the nuclei in the center of the lens fibers. At 15 days, one already finds a small central area completely devoid of nuclei. At 17 days, the centrally located region, devoid of nuclei, accounts for almost one-fifth of the maximum horizontal diameter of the fixed lens. Rapidly the wave of pycnosis extends peripherally. In a 0- to 1-day post-embryonic lens, one-third of the central fiber area lacks nuclei. Study of Feulgen-stained sections revealed an identical picture to those stained with hemalum.

The relative amounts of Feulgen-positive material from the lens epithelial nuclei and sperms were calculated. The comparison of relative amount of DNA from these two cell types revealed that all lens nuclei fall between the diploid and tetraploid category. We did not find any indication of high-ploidy elements in the lens. The same observation was also true for lens fiber nuclei of lenses of four to six days of development. In the older lenses, very few lens fiber nuclei showed relative DNA amounts higher than the diploid content. Most of the nuclei contained DNA which was either equivalent to or less than the diploid amount. A remarkable correlation was obtained by comparison of photographs of individual nuclei and their DNA content. All pycnotic nuclei showed decreasing amounts of DNA. The smallest measurable amount of Feulgen-positive material in few pycnotic nuclei was as low as one-tenth of the normal diploid complement.

Modak *et al.*[2] have shown that DNA synthesis and mitotic activity begin to decrease at an earlier stage of embryonic lens development, in the prospective lens fiber area. From their results and those presented here, one can calculate the shortest interval between the entrance of cells in the terminal cell cycle and final pycnosis of cell nuclei, which amounts to approximately 6½ days. Remarkable series of events follow during this period. These consist of the production of a stable messenger RNA (Reeder and Bell[3]), appearance and intense synthesis of lens-specific proteins (Ikeda and Zwaan[4]), and, finally, decay of rRNA beginning at about eight days of embryonic lens development (Zapisek[5]). It is of interest that the synthesis of the rapidly turning over RNA begins to decrease sharply, just a day before the appearance of pycnotic nuclei.

Our present work clearly shows absence of any high-ploidy elements in the lens population, contrary to the contention of Kvinkhidz,[6] who reported that there are a variety of high-ploidy elements in the chick lens, the highest being equivalent to 32 times the haploid value.

The results reported here clearly demonstrate a progressive loss of DNA from nuclei undergoing pycnosis or degenerative changes. It is conceivable that DNA may be lost from these nuclei either by extrusion of entire or parts of chromosomes or by in situ breakdown of DNA. Due to the absence of mitosis and the condensation of the chromatin, it is impossible to study the former possibility. We have undertaken investigation of the latter possibility. Some of the experiments conducted in this direction are presented in the next communication.

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15.10 STUDIES ON TERMINAL CELL DIFFERENTIATION IN CHICK LENS. II. ANALYSIS OF PRIMING ABILITY AND DENATURABILITY OF DNA FROM VARIOUS CELL POPULATIONS OF DIFFERENTIATING LENS USING CALF THYMUS DNA POLYMERASE

S. P. Modak R. C. von Borstel[1]
F. J. Bollum[2]

DNA polymerase from calf thymus requires single-stranded DNA as a primer for DNA synthesis. Von Borstel, Prescott, and Bollum[3] have demonstrated that nucleotides are incorporated into the newly synthesized DNA polymer in fixed *Euplotes*, HeLa cells, *Nitella*, and mouse blood if the cells were fixed at low pH or allowed to undergo acid denaturation prior to treatment with DNA polymerase. In other words, this method would permit detection of any single-stranded DNA in fixed cell preparation. We have previously shown (Modak *et al.*[4]) that during the terminal differentiation phase, lens fiber cell nuclei undergo pycnotic changes and lose DNA. If DNA is lost by an active breakdown in situ, then at least two possibilities arise, namely, that DNA is broken down into double-stranded pieces or that it first becomes single stranded and then is broken down to smaller single-stranded pieces. In any event, with von Borstel, Prescott, and Bollum's method[3] we have decided to investigate the changes in DNA molecule during the pycnosis. We have also extended the above method to paraffin sections of fixed tissue for the first time.

One-and-one-half-week-old post-embryonic chick lenses were fixed in cold 70% ethanol, embedded in paraffin, and sectioned. Individual sections were mounted on cover slips and then deparaffinized. Similarly, adult chicken blood smears were prepared on cover slips and fixed in cold 70% ethanol. Both chicken blood smears and chick lens sections were divided into several groups. Half of the preparations of each tissue were treated with 0.1 *N* HCl for 30 min at room temperature in order to denature DNA. Both acid-denatured and undenatured cells were incubated with enzyme reaction mixture. A few slides of each group were incubated in the reaction mixture without enzyme, to serve as controls. Enzyme reaction mixture contained dATP-^{3}H, dCTP, dTTP, dGTP, Mg^{2+}, KPi^{2+}, and DNA polymerase. Trace quantities of mercaptoethanol and Versene were also present in the reaction mixture. At the end of treatment, slides were treated in ice-cold 5% TCA in order to remove the acid-soluble radioactivity. After thorough removal of TCA, preparations were coated with liquid emulsion NTB-3 and exposed in dark for two weeks. Autoradiographs were developed in D-19 developer and subsequently counterstained in Mayer's hemalum and mounted in Canada balsam.

Examination of autoradiographs revealed that there did not take place any incorporation of tritium-labeled nucleotides in nuclei of either blood or lens tissue that were fixed in 70% ethanol only. On the other hand, when these cell preparations were treated with HCl prior to the enzyme reaction, DNA polymerase from calf thymus catalyzed a net amount of DNA in both lens and blood nuclei, as indicated by incorporation of radioactivity into the TCA-insoluble component. From these results it is concluded that in both chick lens and adult chicken blood nuclei there is no single-stranded DNA present and that paraffin-embedding procedure did not provoke any denaturation of DNA from lens cell nuclei.

Despite identical acid treatment, various lens cell population demonstrated striking differences in their ability to incorporate radioactivity. Pycnotic nuclei showed highest incorporation of TCA-insoluble radioactivity. To demonstrate these differences we have analyzed various lens cell populations for the frequency of labeling and for their ability to incorporate radioactivity as measured by number of grains per nucleus, as well as grains per unit nuclear area. Figure 15.10.1 shows frequency of labeling in lens cell nuclei and also gives a graphic representation of percentages of labeled nuclei as measured by number of grains over them in the autoradiographs. Clearly, pycnotic nuclei in either phase (pycnosis I or II) have significantly larger proportions of nuclei showing higher grain count than either of lens fiber, annular pad, or lens epithelial cell nuclei. Even within the pycnotic population, nuclei in more advanced stages of pycnosis (pycnosis II) show relatively larger grain-count distribution than those in less

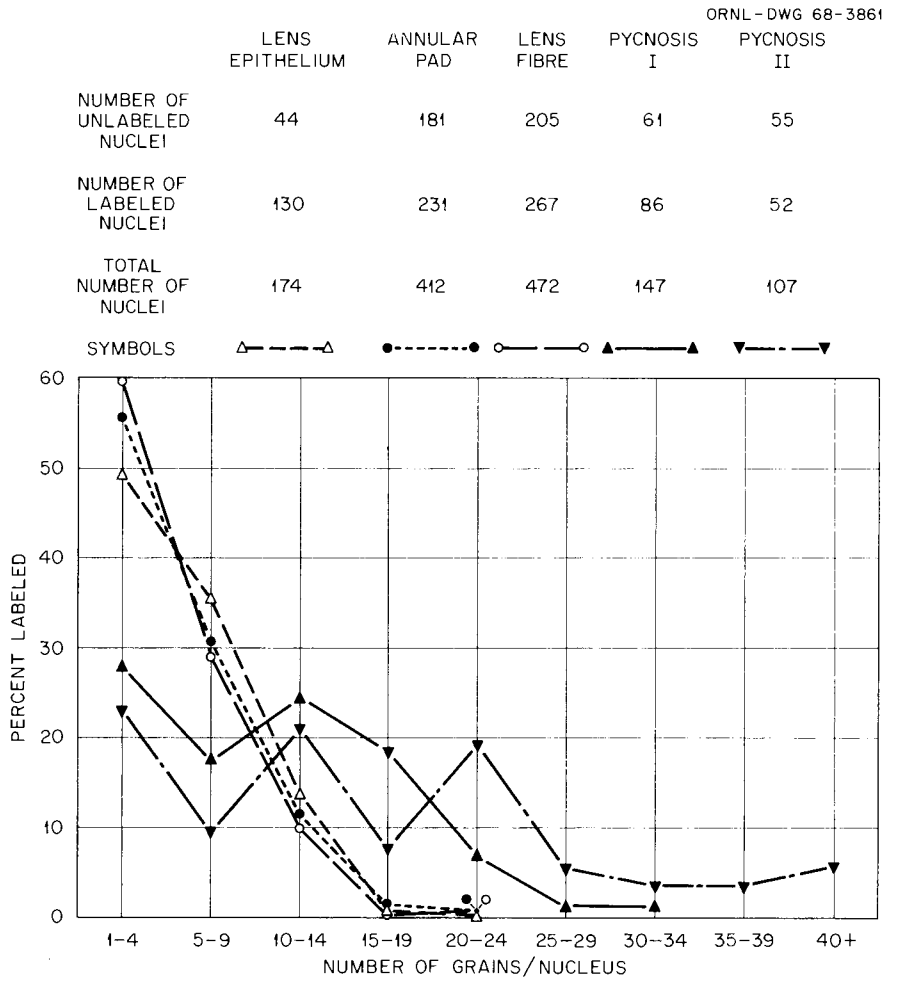

Fig. 15.10.1. Extent of ^{3}H-dNTPs Incorporation in Acid-Denatured Nuclei of Chicken Lens Using Calf-Thymus DNA Polymerase.

advanced stages (pycnosis I). Our data on grains per unit area indicate that much larger differences exist between pycnotic and nonpycnotic nuclei regarding their ability to incorporate ^{3}H-dNTP's with DNA polymerase after acid denaturation. In the light of much smaller amounts of DNA in the pycnotic nuclei as compared with the nonpycnotic population (Modak, Perdue, and Kimball[4]), these results are more significant. In any event, these results have one or more of the following implications: (1) nonpycnotic nuclei contain large molecules of DNA which on acid denaturation would present a relatively smaller number of starting points for DNA replication, (2) nonpycnotic nuclei incorporate smaller amounts of radioactivity due to the relative inhibition of strand separation by histone backbone, (3) DNA in pycnotic nuclei may be composed of a large number of small double-stranded pieces which on denaturation would provide additional sites for the initiation of DNA synthesis, thus incorporating larger radioactivity, and (4) histone backbone itself may be progressively lost during pycnosis, thus rendering DNA more susceptible to acid denaturation.

Considering that all nuclei underwent identical treatment and that relative DNA amounts decrease rapidly during pycnosis, breakdown of DNA as double-stranded pieces during pycnosis seems to us the most plausible explanation for our results. However, the role of histones cannot be neglected. In any event there must take place significant physical changes in the state of DNA during pycnosis. However, these changes do not lead to denaturation of DNA as single-stranded pieces.

We are presently testing the effect of heat and alkali denaturation on the incorporation of deoxyribonucleoside triphosphates using DNA polymerase in fixed preparations of lens cell nuclei.

The important findings of this communication are summarized as follows:

1. As far as the resolution of this method permits, there is no observable priming activity in the DNA of normal or pycnotic nuclei of chicken lens to calf thymus DNA polymerase. Thus DNA does not become single stranded during the pycnosis.
2. This technique can be successfully applied to paraffin sections.
3. DNA from the fixed pycnotic nuclei of lens fiber area are the most sensitive to acid denaturation.
4. Comparing the approximate relative DNA content per nucleus, the higher incorporation of radioactivity with DNA polymerase strongly suggests breakdown of DNA as double-stranded pieces during pycnosis. If this is true, then this method may prove to be a useful tool for detecting a very small proportion of cell nuclei that are undergoing DNA breakdown in a large population of normal cells, which is not always feasible by available biochemical methods.

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15.11 STUDIES ON TERMINAL CELL DIFFERENTIATION IN CHICK LENS. III. ANALYSIS OF THE PRIMING ABILITY OF DNA FROM VARIOUS LENS CELL POPULATIONS USING *MICROCOCCUS* RNA POLYMERASE

S. P. Modak R. C. von Borstel[1]
F. J. Bollum[2]

Von Borstel, Modak, and Bollum[3,4] have shown that alcohol-fixed cell nuclei from chicken blood and other cell preparations are able to incorporate tritium-labeled ribonucleotide triphosphates into newly synthesized RNA using RNA polymerase from *Micrococcus lysodeikticus*. It was clearly demonstrated that RNA synthesized using undenatured blood cell nuclear DNA as primer was loosely bound and readily digestible by RNase. On the other hand, RNA synthesized using acid-denatured DNA as template was much more RNase resistant, indicating the formation of a complex of RNA and DNA. Thus, on the basis of RNase sensitivity of the newly synthesized RNA, one can characterize the nature of DNA molecule.

One-and-one-half-week-old chicken lenses were fixed in cold 70% ethanol and embedded in paraffin. Individual sections were mounted on cover slips and deparaffinized. Sections were divided in two groups: one was treated with 0.1 *N* HCl for 30 min at room temperature to denature DNA and the other one was left untreated. Both denatured and undenatured sections were incubated in RNA polymerase enzyme reaction mixture, with or without the enzyme. Some sections were also treated with DNA polymerase. The composition of RNA polymerase enzyme reaction mixture is indicated in Table 15.11.1.

Sections were thoroughly washed in cold 5% TCA after the enzyme reaction, coated with liquid emulsion NTB-3, and exposed for three to five

Table 15.11.1. Incorporation of ^{3}H-NTP's Using *Micrococcus* RNA Polymerase

Enzyme reaction mixture:	
UTP-^{3}H (2.3 curies/millimole; 1 mc/ml)	0.3 ml
CTP-^{3}H (1.26 curies/millimole; 1 mc/ml)	0.3 ml
ATP (4 m*M*)	0.3 ml
GTP (4 m*M*)	0.3 ml
$MnCl_2$ (4 m*M*)	0.3 ml
Tris-HCl (pH 7.4, to make a final concentration of 0.05 *M*)	0.3 ml
Spermidine (0.8 m*M*)	0.3 ml

Total volume adjusted to 3.25 ml with distilled water.

Removed 0.36 ml for control experiment.

To the remaining reaction mixture, add 0.7 ml of RNA polymerase; total volume, 3.59 ml.

Furnish 30 of enzyme reaction mixture with or without enzyme to each cell preparation

weeks in dark and subsequently developed counterstained in hemalum.

It was found that all cell nuclei, regardless of their previous treatment, incorporated radioactivity into TCA-insoluble component in the presence of RNA polymerase in the reaction mixture. In the undenatured preparations, all cell nuclei incorporated a small amount of nucleoside triphosphates into the newly synthesized RNA. But this RNA was easily digestible by RNase. Important qualitative and quantitative differences in the amount and nature of the incorporated radioactivity exist among various acid-denatured lens cell populations. Acid-denatured epithelial cell nuclei incorporated the smallest amount of isotope with the enzyme. On the other hand, nuclei in the annular pad and the nonpycnotic lens fiber nuclei showed a relatively larger incorporation, a part of which was RNase resistant. Heavy incorporation of RNA precursors was observed in the pycnotic nuclei which was four to five times higher than other lens nuclei. Most of this radioactivity was RNase resistant. Experiments using DNA polymerase gave results which are exact replicas of those described previously (Modak, von Borstel, and Bollum[5]).

Since RNA catalyzed by micrococcal RNA polymerase using double-stranded DNA as template is loosely bound and easily removable by RNase treatment, we have to conclude that there is no detectable amount of single-stranded DNA molecules in the normal or pycnotic nuclei of the differentiating lens.

Lack of significant increase in RNA synthesis by acid-denatured epithelial cell nuclei in the presence of the enzyme points either to their ability to renature DNA or resistance toward acid treatment that we have employed. This result supports the findings reported earlier with DNA polymerase. In spite of DNA loss during pycnosis (Modak, Perdue, and Kimball[6]), DNA of pycnotic nuclei once again suggests the presence of small TCA-insoluble pieces of DNA. The evidence lends support to the probable breakdown of DNA as double-stranded pieces during nuclear degeneration or pycnosis. Undoubtedly, the evidence points to the physical changes in the nature of DNA during pycnosis. We are in the process of studying the breakdown of DNA by alkaline sucrose density gradient centrifugation, the nucleohistone content of lens nuclei, and, finally, the nature and characterization of DNA-degrading enzymes.

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15.12 OBSERVATIONS ON ISOLATED COMPONENTS OF AMPHIBIAN OOCYTE NUCLEOLI

O. L. Miller, Jr. Barbara R. Beatty

A major role for the nucleolus in ribosome biogenesis is now biochemically well documented. However, precise correlation between the known metabolic steps and nucleolar fine structure is lacking. This question is being approached through the study of isolated components of nucleoli from amphibian oocytes.

Mature oocytes of amphibians contain stored ribosomes that are used in protein synthesis during the early stages of embryogenesis. During the growth of this type of oocyte, synthesis of the large 40 to 45s RNA precursor of the 28s and 18s cytoplasmic rRNA's occurs in the hundreds of extrachromosomal nucleoli present in the oocyte nucleus. These nucleoli vary considerably in size, but in thin sections each exhibits a dense fibrous core surrounded by a granular cortex.

The core and cortex components can be separated and dispersed to varying degrees by isolation into several types of media. Electron microscopy of nucleolar cores isolated from *T. viridescens* oocytes into dilute saline demonstrates that each core contains a thin circular fiber. Such circles are disintegrated by DNase, but no breakage occurs with RNase or proteases. The diameter of the thinnest stretches of the fibers suggests that the continuity of the circles is maintained by a single Watson-Crick DNA molecule.

Circles from the larger nucleoli generally are too entangled for accurate contour measurements,

but they appear to be several centimeters long. Of the four smaller circles measured, two were approximately 175 μ and two near 350 μ in circumference. DNA-RNA hybridization studies show that ~0.05% of the haploid genone of *T. viridescens* (45×10^{-12} g of DNA) is complementary to rRNA.[1] The removal of all the rRNA cistrons by a single deletion indicates that these occur within the genome in a single polycistronic cluster.[2] Since only half of the large RNA precursor molecule is utilized in the formation of one each of the 28s and 18s rRNA's and the remaining half apparently degraded,[3] the polycistronic stretch coding for the large precursor molecule should be twice the length of the portion of the genome hybridizing with rRNA. For *T. viridescens* this would amount to about $1\frac{1}{2}$ cm (or ~3333 large precursor molecule cistrons) if the cluster is contained in a single W-C DNA molecule.

If the axes of the circles isolated from the nucleolar cores are continuous DNA molecules and, like lampbrush chromosome loops, can be stretched up to three times their length before breakage occurs, the smallest circles observed could accommodate only 120 cistrons for the large precursor molecule. On the other hand, the larger circles presumably would accommodate at least 10 to 20 times this.

The synthesis of the DNA present in the extrachromosomal nucleoli occurs at the pachytene stage of the oocyte following the replication of the chromosomal DNA.[4] The way in which the nucleolar DNA is replicated and separated from the chromosomes is unknown at present. The observations reported here, however, suggest that the mechanism must give rise to polycistronic clusters both larger and smaller than the nucleolar locus in the genome itself.

Observations on amphibian lampbrush chromosomes suggest that each lateral loop consists of a polycistronic gene being read as a single unit by RNA polymerases,[5] which results in extremely long RNP fibrils and indicates a further breakdown into molecules of smaller sizes. There is a question whether synthesis of the large 40 to 45s precursor of 28s and 18s cytoplasmic rRNA's in the amphibian oocyte nucleolus proceeds in a similar manner or if the precursor molecule is instead produced in single units.

When nucleoli of *Xenopus laevis* oocytes are isolated in 0.1 *M* potassium chloride and transferred to 10^{-4} *M* magnesium acetate, the cortices disperse, allowing the DNA of the cores to unwind with the RNP matrix remaining attached. Electron microscopy of these cores reveals that their axes exhibit periodicity of the attached matrix material, which can best be observed when the DNA axes are stretched (Fig. 15.12.1).

Labeling experiments indicate that the 45s rRNA precursor molecule is synthesized in the nucleolar cores in association with nucleolar DNA and is subsequently transferred to the nucleolar cortex.[6,7] From other studies it is known that the large 45s rRNA precursor molecule is 4.5 μ long.[3] In stretched nucleolar core axes there is a periodicity of alternating matrix-free and matrix-coated DNP areas. Each stretch consisting of one bare region plus one coated region is 6.07 μ in length, the bare region being 2.33 μ and the coated region 3.74 μ. It is probable that the DNA axes are not stretched to capacity and that the 3.74-μ coated area is 4.5 μ long, suggesting that this is the area of the nucleolar DNA axis which codes for the large 45s rRNA precursor molecule.

The stretched matrix fibrils seen on the DNA axis are all significantly shorter (less than 2 μ) than the 4.5-μ length of the 45s molecule (Fig. 15.12.2). If these fibers are the 45s precursor molecules, this suggests that they are read off in single 45s stretches rather than as a polycistronic series of connected 45s molecules.

Electron microscopy of thin sections of amphibian oocyte nucleoli has shown the cortex areas of the nucleoli to be apparently composed of free granules similar in size to the large ribosomal subunit. However, when nucleoli of *Xenopus laevis* are isolated in 0.1 *M* potassium chloride, transferred to 0.5% formalin plus 10^{-4} *M* magnesium acetate, and then centrifuged onto E.M. grids, electron microscopy reveals that the partially dispersed cortices are composed of fibrous networks including granules associated with the probable RNP fibers (Fig. 15.12.3).

A membranous component in nucleoli of *Triturus* oocytes was reported previously.[8] This component has now been detected in oocytes from four more amphibian genera (*Bufo*, *Rana*, *Xenopus*, and *Ambystoma*) and thus appears to be a universal component in such oocyte nucleoli. The component is located within each nucleolus at the core-cortex interface close to the nuclear envelope and usually remains attached to the DNA-containing portion of the core during isolation. Negatively stained, the component appears limited by

Fig. 15.12.1. Stretched DNP Fiber from Nucleolar Core of *Xenopus laevis* Oocyte Exhibiting Periodicity of Attached Matrix Material. 10,500×.

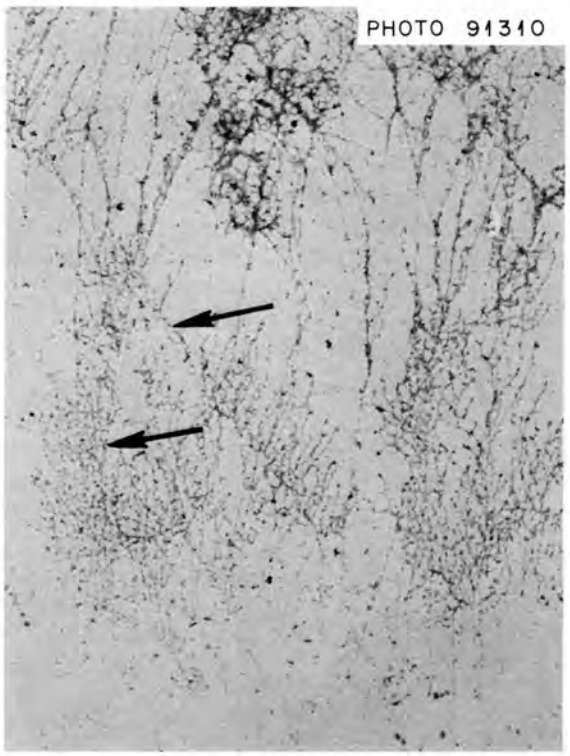

Fig. 15.12.2. Stretched Matrix Fibers Located on Nucleolar Core Axis (Arrows) of *Xenopus laevis* Oocyte. 21,400×.

a 70- to 100-A-thick membrane. It occurs either as a spherical vesicle near 1 μ in diameter, as several smaller vesicles, or as a narrow tubule up to 25 μ long. Autoradiography of isolated cores shows that RNA synthesis is not restricted to the region of the membranous component but occurs simultaneously at many sites on the core. The role of this component in nucleolar metabolism and the question of its presence in nucleoli of other cell types remain undetermined.

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Fig. 15.12.3. Dispersed Nucleolar Cortex of Isolated *Xenopus laevis* Oocyte Exposing Fibrous Network with Associated Granules. 21,400×.

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15.13 SYNAPTINEMAL COMPLEXES IN MEIOTIC CHROMOSOMES

George Gassner III

The hypothesis that synaptinemal complexes (S.C.'s) are present only in chiasmatic meiocytes has been supported primarily by Meyer.[1] Observed with the light microscope, primary meiocytes of *Panorpa nuptialis* G., a North American mecopteran, appear achiasmatic in the male and chiasmatic in the female. Electron micrographs of transverse thin sections reveal that S.C.'s similar in ultrastructure occur in primary meiotic nuclei of both sexes.

Studies by other workers have resulted in the concept that the bipartite feature of the lateral elements of the S.C. represent chromatid separation. The findings of the present investigation support the interpretation that this bipartite feature is allied to homolog rather than chromatid separation. A model is proposed for the plane of homolog separation which differs from previous interpretations by 90°. In this model the plane of homolog separation corresponds to the plane which bisects each lateral element. Lateral elements become increasingly more bipartite until complete separation is accomplished. During this separation, the distance between the lateral elements remains relatively constant in the S.C.'s of *P. nuptialis* and in S.C.'s of all other organisms in which they have been observed.

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15.14 ASPECTS OF VITELLOGENESIS IN THE SOUTH AFRICAN CLAWED TOAD, *XENOPUS LAEVIS*

R. A. Wallace D. W. Jared
Diana Rudack

Vitellogenesis in oviparous vertebrates embraces a number of integrated physiological and developmental phenomena, many of which serve as excellent models for biological processes in general. *Xenopus laevis* lends itself particularly well to experimental manipulations, and thus we undertook a study of several aspects of vitellogenesis in this animal.

Serum Lipophosphoprotein Synthesis by Vitellogenic Females and Estrogen-Treated Males. – Female toads, given 1000 units of human chorionic gonadotropin, ovulated within 24 hr and began to synthesize a lipophosphoprotein which appeared, but did not accumulate, in the serum (physiological

half-life = two days). The period of synthesis lasted about 30 days, after which mature oocytes were once again observed in the ovary. Male toads, given a single dose of 0.1 to 1.0 mg estradiol-17β, also immediately began to synthesize the same lipophosphoprotein. The rate of maximum synthesis and the length of time it took for the maximum to be reached were directly proportional to the amount of estrogen administered. The serum lipophosphoprotein in male toads, however, had a physiological half-life of 40 days, and in extreme cases the accumulated material accounted for more than half of the total serum protein. Females given gonadotropin and subsequently ovariectomized produced a serum lipophosphoprotein with a physiological half-life similar to that of males, presumably because the serum protein is the precursor for the yolk of the developing ovary and, as is the case for the estrogen-treated male, has no place to go.

Characterization of the Serum Lipophosphoprotein. – The serum protein elaborated by the vitellogenic female or estrogen-treated male can be readily isolated in a stable form by chromatography of the serum on TEAE-cellulose. Preliminary measurements indicated that it contains 13% lipid and 1.4% protein phosphorus and has a molecular weight of 4.7×10^5. After butanol extraction or treatment with lipase, phosvitin (the minor yolk protein component of the egg) could be recovered along with an insoluble protein. The latter hypothetically is vitellin, which, in its conjugated form as lipovitellin, is the major component of yolk. Further characterization is in progress in order to definitely define the insoluble component of the serum lipophosphoprotein and the molecular conversions which the latter may undergo during its specific uptake by the developing oocyte and conversion into the crystalline protein yolk platelet.

The Site of Phosvitin Synthesis. – Since phosvitin can be assayed by very specific means and is a component common to egg yolk protein and the serum lipophosphoprotein, we decided to determine its site of synthesis. These experiments involved the culture of various tissues together with an elementary precursor ($^{14}C\text{-}Na_2CO_3$), followed by analysis for labeled phosvitin in the culture medium. The results indicated that only the liver derived from vitellogenic females or estrogen-treated males synthesizes and releases phosvitin into the external medium. All other tissues, including the liver from untreated animals and the vitellogenic ovary, were inactive. Recently, newly synthesized serum lipophosphoprotein has also been identified in the medium for estrogen-treated male liver cultures. Based on these indications, we are attempting now to define the optimum conditions for liver culture so that the hepatic synthesis of lipophosphoprotein and its hormonal control may be studied effectively in vitro.

15.15 CYTOLOGIC AND HISTOCHEMICAL OBSERVATIONS OF OOCYTE MATURATION IN THE ANNELID *ENCHYTRAEUS ALBIDUS*

J. N. Dumont

In a continuing study of the cytologic changes in developing oocytes, representatives of various animal phyla are being investigated from a cytologic point of view. The goal of these comparative investigations is to ascertain basic mechanisms underlying the acquisition of yolk. The present report describes the results from studies of the developing oocytes of the annelid *Enchytraeus albidus*, the common white worm.

The animals were reared in the laboratory at 15°C. Only mature animals, as judged by the presence of large oocytes in the body cavity, were used in this study. Tissues for electron microscopy were fixed in glutaraldehyde-osmium, dehydrated, infiltrated, and embedded in Epon in the usual manner. Enzymatic digestions of thin sections for electron microscopic examination were carried out according to the methods of Monneron and Bernhard.[1] For light microscopy, tissues were fixed with Carnoy's or Bouin's fixatives or in neutral buffered formalin. Various stains, including mercury–bromophenol blue, Heidenhains hematoxylin, Sudan Black B, and PAS, were employed. Lipid extractions with chloroform:methanol (1:1) were done following fixation but prior to embedding.

Some tissue was exposed to an electron-opaque marker (horseradish peroxidase) in order to follow the uptake of material by micropinocytosis. Horseradish peroxidase was applied either (1) by injection into the animal or (2) in medium used for incubation of tissue slices. The results in both cases were identical.

The ovaries of *Enchytraeus* reveal some previously undescribed anatomical features. Syn-

chronously developing oocytes are arranged in packets of eight. All are attached via cytoplasmic bridges to a small central cytoplasmic mass. The cell membrane of the bridges is modified by the presence of a dense material along its inner surface. The cytoplasmic bridge is retained until near the final stages of vitellogenesis, when it breaks and the oocytes float free in the body cavity. Cytoplasmic continuity between all the oocytes of the packet undoubtedly plays a role in keeping development synchronous within the packet of cells.

The ooplasm of the early oocyte is characterized by the presence of many ribosomes (polysomes), a few cisternae of smooth and rough endoplasmic reticulum, small Golgi complexes, and mitochondria. These organelles are accumulated at the apical end of the cell opposite the cytoplasmic bridge. Many of the mitochondria of oocytes in early stages of differentiation present unique profiles. Although small, they are constricted for some distance along their long axis and thus appear dumbbell-shaped. In addition, many of the dumbbell-shaped mitochondria are curved into a crescent shape. Since the number of mitochondria increases during oocyte development, it is possible that these configurations represent stages of mitochondrial division.

As the oocyte matures, the first sign of vitellogenic activity is the appearance of lipid droplets scattered throughout the ooplasm. Electron microscopic examination of oocytes at this time reveals, in addition to the lipid droplets, an increase in the quantity of smooth endoplasmic reticulum, many large scattered mitochondria, and an increase in the number and size of the Golgi complexes. Also appearing in the ooplasm near the Golgi complexes are small dense bodies which represent the initial accumulations of yolk. As these yolk bodies mature, they come to consist of two distinct parts: (1) relatively dense spherical bodies (core bodies) which are surrounded by (2) a much more electron-dense matrix. The entire yolk body is bounded by a unit membrane. The number of core bodies within a single yolk body varies – the largest yolk bodies may contain up to six core bodies.

Observations regarding the origin of yolk indicate that a portion of the yolk is formed by the oocyte and involves synthesis and/or packaging by the Golgi complexes. Similar observations have been made regarding the origin of yolk in the oocytes of *Limulus*.[2] Micropinocytotic activity increases gradually throughout vitellogenesis. The micropinocytotic vesicles fuse in the peripheral ooplasm with developing yolk bodies which may or may not contain core bodies. The density of the material derived from micropinocytosis resembles the matrix. That extraoocytic material is incorporated by micropinocytosis has been demonstrated by following the uptake of horseradish peroxidase. Electron microscopic analysis shows that peroxidase accumulates in the matrix of the developing yolk bodies; however, it has never been found in the core bodies. Thus, material incorporated by micropinocytosis is deposited in the matrix of the yolk body.

Regarding histochemical procedures, staining with Sudan Black revealed the presence of lipid in the matrix but not in the core bodies. PAS staining indicated a strong reaction in the matrix but only a weak reaction in the core bodies. However, lipid extraction abolished both the Sudan Black staining and the PAS-positive reaction in the matrix but did not abolish the PAS-positive reaction in the core bodies. Mercury–bromophenol blue reveals a strong reaction for proteins in the core bodies and a weak reaction in the matrix. Enzymatic digestions of thin sections show that the core bodies are removed by α-amylase and pepsin. Although the matrix appears somewhat degraded after this treatment, it nevertheless remains even after 12 hr of incubation. Pronase, on the other hand, removes the matrix without degrading the core bodies. Hyaluronidase is ineffective in removing either component. These enzymatic procedures were further confirmed by digestions using paraffin-sectioned material. Electron microscopic examination of tissue extracted with chloroform:methanol shows that a portion of the matrix is removed. What remain after extraction are filamentous strands of material, which probably represent the protein moiety of the matrix. Lipid extraction does not affect the core bodies.

The evidence obtained in this study suggests that the core bodies are relatively rich in carbohydrates and the matrix in lipid. Peroxidase tracer studies indicate that at least part of the matrix is derived from extraoocytic sources and incorporated into the oocyte by micropinocytosis. The carbohydrate-rich core bodies, on the other hand, may be synthesized and/or packaged by the Golgi complexes of the oocyte. During vitellogenesis yolk from both sources is combined to form the mature yolk body.

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15.16 THE ORIGIN AND FORMATION OF CORTICAL GRANULES IN THE OOCYTES OF AMPHIOXUS, *BRANCHIOSTOMA CARIBAEUM*

J. N. Dumont

The importance of cortical granules lies in their ability to form a nearly impervious coat over the surface of the egg during and immediately following fertilization. In amphioxus the cortical granules of mature eggs are aligned in a layer approximately 5 μ deep, just beneath the oolemma. The cortical granules give a weak positive reaction for polysaccharides and proteins when stained by the PAS procedure and mercury–bromophenol blue respectively. Preparations stained with Alcian Blue, however, show an intense positive staining reaction in the cortical granules, which indicates the presence of acid mucopolysaccharides. In younger oocytes small acid mucopolysaccharide deposits are found scattered throughout the ooplasm. These deposits enlarge and migrate to the periphery of the cell during maturation and contribute to the formation of the cortical granules.

Electron micrographs of young oocytes reveal small Golgi elements consisting of stacks of about five lamellae scattered throughout the ooplasm. During maturation these organelles enlarge, and those lamellae located at the concave surface of the Golgi begin to dilate either distally or along their entire lengths. As a result relatively large vacuolar structures, many of which contain a very fine particulate material, are formed. These subsequently fuse with others and migrate to the periphery of the oocyte, where they become the cortical granules of the mature egg. It has been reported that cortical granule formation in amphioxus is related to micropinocytotic activity of the oocyte.[1] However, information obtained from this study indicates that the cortical granules take their origin from the Golgi complexes and that their formation is unrelated to micropinocytosis.

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15.17 CYTOLOGIC CHANGES ASSOCIATED WITH SPERMIOGENESIS IN *RHYNCHOSCIARA SP.*

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Ellen Mattingly[1]

The sciarid fly *Rhynchosciara sp.* has within the past few years become an important and useful organism for cytogenetic studies. Of particular interest in this laboratory are certain changes that occur during spermiogenesis, especially those related to the mitochondria and the "limited" chromosome. *Rhynchosciara* is especially suited for these developmental studies, since larvae of each group develop synchronously.

By 30 days after the larvae hatch, DNA synthesis in the spermatogonia ceases. However, scattered throughout the testes are large polytene cells which do continue to synthesize DNA. Electron micrographs show that cytoplasmic processes of these large polytene cells ramify into many of the interstices between adjacent spermatogonia. Close associations between these large cells and the spermatogonia or spermatids similar to that found between Sertoli cells and spermatids, as, for example, in the mammals, have not been observed.

Well-defined cytoplasmic bridges provide continuity between as many as four neighboring spermatogonia. These interconnections are undoubtedly related to the high degree of developmental synchrony found among groups of cells in the testes.

After DNA synthesis ceases and mitosis is completed, spermatogonial nuclei revert to the interphase appearance, and small "granules" appear in the cytoplasm. These increase in size and number during the period between the end of mitotic divisions and the beginning of meiosis. Electron microscope examination of cells during this phase reveals that the "granules" are mitochondria which contain a large droplet of material in their matrices. By four days after hatching, the mitochondria are all located in a circumnuclear position and under phase-contrast optics appear refractile. Beginning with spermatid elongation,

these organelles fuse. In the mature sperm the material is located within one large mitochondrion and lies in a mass which occupies a tract along the entire length of the spermatozoon. In the spermatheca of the female the spermatozoa shed this material. Its function remains obscure.

Histochemically, the "granules" give a positive Janus Green B reaction, a test that is characteristic of mitochondria. They are negative for lipid, do not stain with Feulgen, and give a weak polysaccharide-positive reaction. They also stain intensely with protein stains and with pyronine Y; the latter reaction is not changed by prior extraction with RNase.

During the time following mitosis and when the cytoplasmic "granules" are accumulating, the limited chromosome becomes pronounced adjacent to the nuclear membrane. Accumulations of a finely granular electron-dense material are seen around the periphery of this chromosome. The nature of this material is now being investigated.

Reference

[1]Cytology and Genetics section.

15.18 ISOLATION OF HEMOCYANIN FROM HEMOLYMPH OF *CALLINECTES SAPIDUS*

Marilyn S. Kerr

Hemocyanin, the copper-containing respiratory pigment of many Arthropoda and Mollusca, has been a favorite protein for chemical, physical, and serological studies since Ericksson-Quensel and Svedberg first calculated the molecular weights of different types of hemocyanin from sedimentation data.[1] Hemocyanin (HCy) is not contained in cells but is a plasma protein, and generally, in most studies of the HCy molecule, it has been assumed to be the major constituent if not the only plasma protein present in the hemolymph. It is distressing to question the results of otherwise excellent studies, but one wonders, when the above assumption has been made, if investigators were studying only HCy.

Little has been published concerning the hemolymph proteins, except HCy, of molluscan forms. This might be unique if, in its collective sera, HCy is the only protein present. The serum proteins in crustaceans and in *Limulus polyphemus* vary quantitatively and qualitatively. In the blue crab, *Callinectes sapidus*, HCy is always present in the hemolymph and, in some individuals, may constitute as much as 97% of the serum protein; in other individuals, it may be only one of at least five (possibly more) constituents and may constitute as little as 45% of the total serum protein. This report outlines a simple method for isolating HCy from hemolymph serum of the blue crab.

DEAE-cellulose (120 to 200 mesh) was processed and packed essentially as described by Peterson and Sober[2] and Wallace.[3] The chromatographic procedure, including washing of the columns, gradient apparatus, and flow rate, is that developed by Wallace.[3] A 29 × 1.9 cm column was equilibrated with 0.05 *M* ammonium bicarbonate, pH 7.8. A concave gradient of sodium chloride and magnesium chloride was used to separate the proteins. The first two bottles contained 0.05 *M* NH_4HCO_3 and the third bottle contained 0.05 *M* NH_4HCO_3, 0.5 *M* NaCl, and 2.0 m*M* $MgCl_2$. Ammonium bicarbonate was used to facilitate concentration of the low-molecular-weight, protein-like material eluted from the column in the initial void volume. The relatively low pH and the presence of Mg^{2+} prevent dissociation of the HCy; 2.0 m*M* Mg^{2+} is essential to avoid precipitation of the HCy during concentration of the protein after elution from the column.

Figure 15.18.1 shows a tracing of the optical density (280 mμ) recorded during the separation of proteins in an undialyzed 1.5-ml sample of adult female *Callinectes sapidus* serum. The gradient used had a total volume of 900 ml.

The protein-like material eluted from the column in the void volume can be removed by dialysis of the hemolymph sample against 0.05 *M* NH_4HCO_3 prior to chromatography. More of this material is present in serum than in plasma from the same blue crab. This fact and other preliminary observations indicate that this material, apparently released by the hemocytes, may be involved in clotting of hemolymph. The proteins eluted by the gradient, represented in Fig. 15.18.1 as groups 1, 2, and 3, were concentrated by vacuum ultrafiltration,[3] dialyzed against phosphate buffer containing 2 m*M* $MgCl_2$, pH 7.4, and analyzed by starch gel electrophoresis and immunodiffusion. The protein represented by the first peak is most striking in its quantitative variation among individual sera. This protein binds ^{59}Fe and is

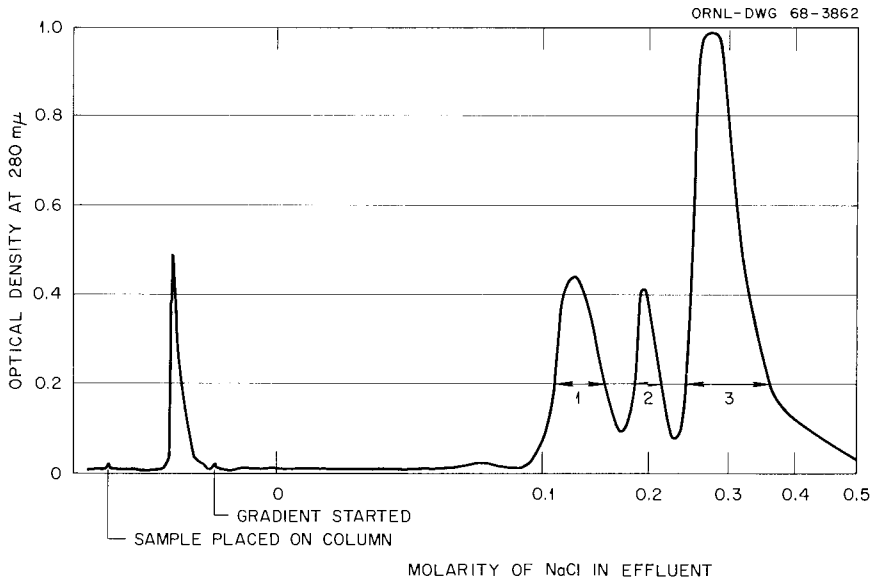

Fig. 15.18.1. DEAE-Cellulose Chromatography of Hemolymph Serum from the Blue Crab.

associated with amylase activity (these two properties have been inseparable in all protein-separatory techniques tried to date), but nothing specific can be said about its function. The protein represented by the second peak is a copper-bearing protein called "slow hemocyanin" by Manwell and Baker[4] and found to be present only in sera from females, but not all females (Horn and Kerr, unpublished). It does not appear to dissociate with increasing pH, and nothing is known about its oxygen-combining properties other than that it, like HCy, acts as a peroxidase in staining reactions after electrophoresis in starch gel or acrylamide. The third peak represents HCy. This is easily distinguished by the blue color of the column effluent. The copper: protein ratio is approximately 0.2:100. The protein dissociates into characteristic subunits when the pH is increased or during electrophoresis in borate buffers.

This chromatographic technique is not recommended for the separation of all hemolymph serum proteins. Several different buffer systems have failed to elute a serum lipovitellin from DEAE-cellulose. The method described above may be useful to other investigators who wish to prepare hemocyanin.

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15.19 INCORPORATION OF LEUCINE INTO HEMOCYANIN DURING SHORT-TERM ORGAN CULTURE OF HEMOCYTES AND HEPATOPANCREAS FROM THE BLUE CRAB

Marilyn S. Kerr

In an earlier attempt to label a serum lipoprotein as a means of determining whether or not it is a precursor to lipovitellin, female blue crabs were fed or injected with L-leucine-1-^{3}H or L-leucine-1-^{14}C. Although no lipoprotein was present in any of the sera from these crabs, all of the other serum proteins appeared to be labeled within 6 to 10 hr

after administration of the radioactive leucine. Autoradiography of tissues from these animals failed to reveal any specificity in localization. For this reason, it was decided to use organ culture techniques and to try to analyze proteins released into the culture medium.

Blue crabs were anesthetized by placing them in ice until reflex actions in response to touch disappeared. A periopod was washed in 95% ethanol and cut off near the base. Hemolymph was allowed to drip freely into gently swirling tissue media. Tissues (hepatopancreas, ovary, heart, and leg muscle) were excised, placed immediately in sterile phosphate-buffered saline, minced, washed in three changes of the buffered saline, blotted with filter paper, and placed in the incubation media. Three different media, obtained from Grand Island Biological Company, Grand Island, New York, have been used for the incubation of these tissues: phosphate-buffered saline (1X) and minimum essential medium (without leucine) with Earle's salts (both designed for the culture of mammalian tissues), and Grace's insect T.C. medium. All media contained 1.0 m*M* sodium pyruvate and L-leucine-1-^{14}C (10 μc/ml). Tissues and hemolymph from the same animals were cultured in all three media.

Sampling at intervals during incubation at room temperature (20 $\pm$ 0.5°C) showed that the rates of ^{14}C incorporation into total protein were extremely variable. Incorporation in Grace's insect T.C. medium was slight. Tissues incubated in minimum essential medium showed the greatest incorporation. After incubation, the hemocytes and tissue fragments were removed by centrifugation. A 1.0-ml aliquot of each supernatant solution was mixed with 1.0 ml of hemolymph serum and chromatographed on DEAE-cellulose. Analyses of fractions resulting from chromatography of media in which hemocytes or hepatopancreas had been cultured revealed labeled hemocyanin.

The possibility that hemocyanin is synthesized in both the hemocytes and the hepatopancreas seems remote. The results obtained with hepatopancreas tissue might be explained by failure to remove all of the hemocytes before placing it in the incubation media. Morphologically, the hepatopancreas is complex, consisting of minute finger-like projections closely packed together, creating folds in which hemocytes might be trapped.

After the initial experiment using whole hemolymph resulted in labeled hemocyanin, control studies in succeeding experiments included the culture of serum in the same manner as the other tissues. No radioactivity was incorporated into the protein. Therefore, if the incorporation of leucine into hemocyanin by whole hemolymph cultures is merely an exchange of leucine in the hemocyanin for leucine-1-^{14}C of the culture medium, the hemocytes must play an integral role in this turnover. A definitive experiment would be incubation of hemocytes alone. Attempts to isolate these cells intact have been somewhat less than successful. Hemocytes are involved in hemolymph clotting (the mechanisms of which are unknown in crustaceans), and the clotting time of crustacean hemolymph is instantaneous when compared with that of mammalian blood. The best means found yet to inhibit clotting is to draw hemolymph into a syringe containing concentrated EDTA to a volume having a minimal EDTA concentration of 0.6%. The clotting reaction can be retarded by chilling the crab before bleeding and by collecting the hemolymph in a previously chilled plastic centrifuge tube. In either case, after gentle centrifugation to bring down the cells, the hemocytes tend to clump and stick to the bottom of the centrifuge tube. Attempts to collect the cells on Millipore filters have resulted in no greater success so far. The cells are disrupted when they hit the filter, resulting in coagulation to the extent that the diluted serum proteins cannot be pulled through the filter.

Future work will be directed toward isolation of the hemocytes and investigation of their synthetic capacities.

16. Biochemistry of Cell Differentiation

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16.1 THE SYNTHESIS OF LENS PROTEINS BY EPITHELIAL CELLS IN TISSUE CULTURE

John Papaconstantinou J. W. Carr

Epithelial cells from embryonic calf and adult lenses have now been successfully maintained in continuous culture for a period of two years. One of the questions we have been concerned with is the degree of stability of these cells in culture with respect to their ability to synthesize the tissue-specific lens protein (α-, β-, and γ-crystallins). Experiments have been carried out to determine whether α-, β-, and γ-crystallins are synthesized by cells in primary culture, 9th subculture (90 days), 14th subculture (140 days), 25th subculture (250 days), and 44th subculture (440 days). At the end of each culture period, the cells were washed with Hank's salts and were incubated for 6 hr in a medium containing ^{3}H-amino acids in Hank's salts and 30% calf serum. After the incubation period the cells were again washed and removed from the glass surface by dispersing with viocase. The dispersed cells were packed down by centrifugation and resuspended in a homogenate of calf lens fiber cells which had been labeled with ^{14}C-amino acids. Thus the fiber cell homogenate contained ^{14}C-labeled α-, β-, and γ-crystallins, which were used as carrier proteins for the ^{3}H-labeled proteins of the cultured cells. The cells were homogenized in 0.005 *M* sodium phosphate buffer, pH 7, and dialyzed exhaustively against the same buffer.

The α-, β-, and γ-crystallins were fractionated on DEAE columns. Each fraction was analyzed for optical density at 280 mμ to identify the crystallins as they were eluted from the column. These fractions were also analyzed for ^{14}C and ^{3}H by a double-labeled counting procedure using a liquid scintillation counter. The ^{14}C counts could, therefore, be used to detect the radioactive proteins synthesized by fiber cells, and the ^{3}H counts could be used to detect the proteins synthesized by the cells in tissue culture. In these experiments special attention was given to the elution properties of the ^{14}C- and ^{3}H-labeled proteins to determine whether or not these counts were superimposed as they were eluted from the column. In order to determine any slight variation in elution properties, a gradient elution system was used in these experiments. The results of this experiment showed that: (1) the lens cells in tissue culture synthesize α-, β-, and γ-crystallins, (2) there was

no difference between the ^{14}C and ^{3}H peaks, and (3) in all cases both ^{14}C- and ^{3}H-labeled peaks followed the optical density pattern (280 mμ). (Similar results were obtained when a stepwise elution system was used.)

The Synthesis of α-, β-, and γ-Crystallins by Cells in Tissue Culture. – In all of the cultures studied, the DEAE-cellulose fractionation procedure indicated that α-, β-, and γ-crystallin synthesis is actively going on. There did not appear to be any significant differences in the chromatographic properties of the crystallins from calf fiber cells and from the cultured epithelial cells.

α-Crystallin Synthesis. – One of the interesting changes that did occur during the continued culturing of epithelial cells is the increase in synthesis of the α-crystallins. Fractionation of the α-crystallins into their five distinct proteins showed that the synthesis of α-crystallins *f*, *g*, *h*, and *i* is much greater than the increase seen in α-crystallin peak *e*.[1] Since the α-crystallins differ from each other with respect to their quantitative content of specific subunits, this preferential stimulation of protein synthesis may be due to an increase in synthesis of specific subunits. The rate of synthesis of these subunits from noncultured and cultured epithelial cells is now being studied.

Antiserum to the purified α-crystallins was prepared, and each of the cultures was further analyzed by immunofluorescent techniques. There was a strong fluorescence in all of these cultures. Thus α-crystallins are synthesized by 44th subculture cells which have been in culture for 440 days.

β-Crystallin Synthesis. – The synthesis of β-crystallins was also studied by the chromatographic and immunofluorescent techniques. Again, all cells up to the 44th subculture showed a strong immunofluorescent response, indicating that the proteins are present in these cells. In addition, the proteins from cultured cells showed similar chromatographic properties (DEAE-cellulose) to the β-crystallins from fiber cells. There did not, however, appear to be a corresponding increase in β-crystallin synthesis as was seen with the α-crystallin.

γ-Crystallin Synthesis. – In the intact calf and adult lens, γ-crystallin synthesis is specifically found in the region of cellular elongation and in the fiber cells. The immunofluorescent data in this series of experiments show that there is a large amount of γ-crystallins present throughout the cultured epithelial cells of primary explants. Furthermore, γ-crystallin fluorescence can be seen in all subsequent subcultures by the immunofluorescent procedure, and the fact that they are being synthesized was indicated from the fractionation of radioactive proteins from the cultured cells. Thus the immunofluorescence data indicate that γ-crystallin synthesis can be induced in epithelial cells when they are removed from the lens and placed in culture. Since there are several γ-crystallins making up this group of proteins, experiments were carried out to determine whether all of these crystallins as seen in lens fiber cell synthesis are synthesized by the cells in tissue culture. To carry out these experiments the γ-crystallins were isolated from the other proteins on a DEAE column at pH 7. The eluted γ-crystallins were precipitated down with ammonium sulfate and redissolved in 0.01 *M* Tris, pH 10. A DEAE column was also equilibrated with 0.01 *M* Tris, pH 10, and the column was charged with the γ-crystallins. The individual γ-crystallins were then eluted from the column by the stepwise addition of Tris HCl buffers in the following concentrations: (1) 0.02 *M*, pH 9; (2) 0.04 *M*, pH 8.6; (3) 0.06 *M*, pH 8.2; (4) 0.08 *M*, pH 7.6; (5) 0.1 *M*, pH 7.2. Using this elution system the γ-crystallins can be resolved into five peaks. Each fraction was analyzed for ^{14}C and ^{3}H proteins. Again it was found that the ^{14}C and ^{3}H peaks completely overlapped, and both ^{14}C and ^{3}H peaks coincided with the optical density peaks. These data indicate, therefore, that the cells in culture can synthesize γ-crystallins similar to those found in the fiber cells.

This extensive series of experiments strongly indicates that cultured lens epithelial cells are extremely stable in that they maintain the ability to synthesize crystallin proteins after extensive subculturing. In this respect it should also be pointed out that these cells do not lose their morphological characteristics throughout this culture period; that is, they retain the morphological characteristics of the epithelial cell. Since the epithelial cells initiate the synthesis of γ-crystallins very soon after they are placed in culture, these observations indicate that γ-crystallin synthesis in the lens is regulated so that it occurs only in the region of cellular elongation and in fiber cells. When these cells are placed in culture, this regulation is released. Further studies on the mechanism of "induction" of γ-crystallin synthesis in culture are now being carried out.

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16.2 THE REGULATION OF RIBOSOMAL RNA SYNTHESIS IN BOVINE LENS EPITHELIAL CELLS AND FIBER CELLS

John Papaconstantinou Emilia M. Julku

In a continuation of our studies on the stabilization of mRNA during fiber cell differentiation and in the adult epithelial cells, experiments have been carried out to determine the nature of rapidly turning over RNA in these cells. One of the main objectives of these studies is to determine the properties of ribosomal RNA synthesis and the mechanisms of ribosomal assembly in cells which have undergone terminal cellular differentiation, such as the fiber cell, and in adult epithelial cells which are in an extended stationary or G_1 phase of the cell cycle. Both of these cell types show similar properties in that they carry out protein synthesis on stable templates,[1,2] but they differ from each other in that the fiber cell has lost the ability to replicate, whereas the epithelial cell can be induced to go into division. Thus the properties of ribosomal RNA synthesis and degradation in the cells having stable templates (epithelial cells in a stationary phase) and rapidly turning over templates (dividing epithelial cells) have been studied. In these experiments lenses were labeled with either guanidine or uridine to follow overall RNA synthesis and with [$^{14}CH_3$]-methionine to follow the synthesis of ribosomal precursor RNA, as well as the formation of 28s and 18s ribosomal RNA components. The epithelial and fiber cells were separated at the end of each experiment, and each cell type was phenol-extracted. The RNA was fractionated on sucrose density gradients, methylated albumin kieselguhr (MAK), or acrylamide gel electrophoresis to follow the pathway of synthesis and degradiation of ribosomal RNA. In all experiments reported, the lenses were pulse-labeled for 3 hr with RNA precursors and chased for 3 hr with actinomycin D.

Analysis of pulse-labeled RNA by sucrose density gradients showed major radioactive peaks in the 45s, 30s, 20s, and 7 to 10s regions. The radioactive methyl groups from methionine were found to follow the 45s and 30s regions closely. In no case could we find significant radioactivity coincident with the 28s and 18s ribosomal RNA peaks. There were also regions of radioactivity greater than 50s detected by short-term gradient centrifugation. These regions did not show any methylation, indicating that they are not ribosomal. Thus by following the incorporation of methyl groups, we were able to locate the ribosomal precursor RNA's in both sucrose gradients and MAK columns.

In an attempt to determine the amount of rapidly turning over RNA going into the assembly of the ribosome, that is, into 28s and 18s, all RNA synthesis was stopped by the addition of actinomycin D to the incubation medium after a 3-hr incubation with the radioactive precursors. An analysis of the epithelial cell RNA chased with actinomycin D showed that only a negligible amount of the methylated 45s and 30s RNA appeared in the 28s and 18s RNA. Similar observations were made with the fiber cell RNA analyses. These data indicate that although 45s and 30s RNA are synthesized (especially by the epithelial cells) this RNA was not being incorporated into the ribosome. Our data indicate that the regulation of ribosomal assembly does not involve the cessation of rRNA synthesis and that one possible site of regulation may be the inhibition of formation of 28s and 18s. Under these conditions the ribosomal RNA precursors, that is, 45s to 30s are synthesized and the 30s is degraded instead of being incorporated in the ribosome.

Since the epithelial cells can be induced to divide by placing the lenses into organ culture, similar experiments as those described above were carried out with epithelial cells from cultured lenses. These analyses showed that there was an overall increase in RNA synthesis after three days in culture. In addition, after treatment with actinomycin D, it was found that the majority of the radioactive RNA was chased into the 28s and 18s RNA. This was also observed with the radioactive methyl groups. From these experiments we conclude that nonstationary cells can complete the processing of ribosomal RNA, which is ultimately found in the assembled ribosome as 28s and 18s species. Thus a major difference between the epithelial cells in a stationary phase and those undergoing replication lies in the ability of the dividing cells to produce 28s and 18s RNA. It appears, therefore, that a major regulatory step in ribosomal assembly is the reactions involving the conversion of 30s RNA to 28s and 18s RNA. Ex-

periments are now being carried out to determine the mechanism for the regulation of 28s and 18s RNA formation.

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16.3 AN INCREASE IN THE COMPLEXITY OF α-CRYSTALLINS DURING LENS CELL DIFFERENTIATION

Winifred G. Palmer John Papaconstantinou

Preliminary studies of the changes in crystallin proteins occurring during lens development have indicated that the α-crystallins of fetal, calf, and adult lenses differ in their chromatographic properties when fractionated on DEAE-cellulose. However, the α-crystallins isolated from lenses of different stages of development are immunochemically identical. The chromatographic differences between them can now be correlated to the changes in the subunit content of the protein.

The procedures for isolation of α-crystallins and subsequent fractionation into their subunits have been reported elsewhere.[1] Chromatography of adult cortex α-crystallin by DEAE-cellulose in the presence of 7 *M* urea fractionates the protein into seven subunits. To facilitate comparisons between the proteins of different age lenses, the subunit peaks from the columns have been divided into three groups; region I contains subunits 1 and 2, region II contains subunits 3 through 6, and region III contains subunit 7.

It can be seen in Table 16.3.1 that there are significant differences in the subunit composition of the α-crystallins extracted from lens cortices at the three different stages of development. Subunits 1, 2, 3, and 4 are present in all of the α-crystallins studied. Subunit 7 is present in very small amounts in the fetal and calf fiber cells. There is a large increase in the amount of this subunit in the adult cortex α-crystallin. The presence of only small amounts of subunit 7 in the adult epithelium may indicate that the formation of this polypeptide chain is associated with fibrogenesis in the adult lens. Further differences between the subunit compositions of fetal and adult α-crystallins are found in subunit peak 5 (region II), which is entirely absent from the fetal protein and apparently is not synthesized until after birth. These data indicate that the development of the lens is accompanied by an increase in the complexity of α-crystallins. The relative rates of synthesis of the various subunits also change during development.

Table 16.3.1. The Subunit Composition of the α-Crystallins from Lenses at Different Stages of Development

Age	Percent of Each Subunit Region in Total Protein		
	I	II	III
4-Month embryo cortex	57	39.6	3.4
4½-Month embryo cortex	43.4	52.1	4.4
Calf cortex	22.6	73.8	3.5
Adult cortex	18.2	63.5	18.3
Adult nucleus	28.9	64.2	6.9
Adult epithelium	26.4	63.9	9.7

Experiments are now being carried out to compare the half-lives of the messenger RNA's responsible for the synthesis of the different subunits. Preliminary experiments indicate that the half-lives vary considerably between the subunits.

Reference

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16.4 AN IMMUNOFLUORESCENT ANALYSIS OF THE DISTRIBUTION OF α-CRYSTALLINS IN EMBRYONIC AND ADULT BOVINE LENS CELLS

R. E. Shore Winifred G. Palmer
John Papaconstantinou

As a result of our recent studies on the immunochemical, biochemical, and physicochemical anal-

yses of the α-crystallins of the bovine lens, we have been able to prepare a specific antiserum to one of the five α-crystallins.[1] This specific antiserum was used to study the distribution of α-crystallins in fetal and adult lens epithelial and fiber cells through the use of immunofluorescent techniques. It is the purpose of this project to determine whether the distribution of the α-crystallins is altered during the growth of the lens and to establish cytological evidence for the regulation of synthesis of this group of structural proteins. The immunofluorescent analyses have shown that early in gestation α-crystallins are evenly distributed throughout the central, peripheral, and equatorial regions of the epithelial cell layer. Strong fluorescence was also seen in the fiber cells of these lenses.

At the end of the first half of the gestation period (160 days), α-crystallin is no longer found throughout the entire epithelium. In the central epithelial region of lenses from late fetuses, the intensity of fluorescence decreases markedly, while a significant amount of fluorescence can still be detected in the epithelial cells of the peripheral region and in the fiber cells. In adult lenses the epithelial cells of the entire central region are devoid of fluorescence. This loss of fluorescence extends to the peripheral region just anterior to the equator, where the process of cellular elongation or fibrogenesis is initiated. These immunofluorescent analyses indicate, therefore, that there is a continued loss of detectable α-crystallin, starting from the cells of the central region and extending progressively to the cells of the peripheral region.

An interesting difference between the intracellular distribution of the α-crystallins and the β- and γ-crystallins in epithelial and fiber cells has also been found. Initial studies with the β- and γ-crystallins have shown that these proteins are present in the nuclei of elongating cells and fiber cells. With respect to the α-crystallins, no fluorescence could be detected in any nuclei at all ages studied. Thus the α-crystallins are specifically localized in the cytoplasm of epithelial cells and fiber cells, whereas the β- and γ-crystallins can ultimately be localized throughout the cytoplasm and nucleus of these cells.

In addition to the fluorescent analyses described above, a series of sections from fetal and adult lenses were examined by dark-field illumination. Under these conditions the granularity of the cytoplasm can easily be seen. The dark-field illumination pictures of the embryonic cells show a high degree of granularity throughout the entire epithelial cell layer. Similar photographs of the epithelial cells in adult lenses show that there is a significant loss of granularity in the central region cells. A comparison of the fluorescent and dark-field sections has shown that the cells which have lost their granularity are also the ones which cease to show fluorescence. Furthermore, in the peripheral region of the adult epithelial cells, there is an increase in granularity, and it is in this region that the fluorescence is retained. These data indicate that the α-crystallins may be associated with these cytoplasmic granules and that the loss of fluorescence may be due to the complete breakdown of α-crystallins when the granules are lost. Similar preliminary experiments in which conjugated antibody to γ-crystallins has been used show that γ-crystallin fluorescence also follows the granularity of the cell. Thus the γ-crystallins may also be associated with these granules. These observations indicate that the structural proteins of the lens, that is, α-, β-, and γ-crystallins, may be complexed within the cytoplasm as granules and further indicate the existence of a highly organized structure in the cytoplasm.

Finally, we conclude from these studies that the regulation of synthesis and degradation of the α-crystallins in the epithelial cells may be a function of the age of the cells and possibly of their mitotic activity. We suggest the latter, that is, mitotic activity, because of the well-known phenomenon that the epithelial cells of adult vertebrate lenses are in an extended G_1 (stationary) phase and will only go into mitosis by physical or chemical stimulation. Thus although these cells have not lost their ability to divide, the mitotic index of the adult epithelial cells is negligible, and they are in this extended G_1 phase indefinitely. Further experiments on the regulation of synthesis and degradation of the α-crystallins in stationary and dividing epithelial cells are now in progress.

Reference

[1] W. G. Palmer and J. Papaconstantinou, *Biochemistry* **7**, 243 (1968).

16.5 THE STABILIZATION OF PROTEIN-SYNTHESIZING TEMPLATES IN BOVINE LENS

J. A. Stewart John Papaconstantinou

We have previously shown that the stabilization of mRNA is a process occurring in the lens during (1) the differentiation of the epithelial cell to fiber cells and (2) in the adult epithelial cells, when the mitotic index is negligible and the cells have entered a stationary or extended G_1 phase of the cell cycle. Since the adult lens epithelial cells have not lost their ability to divide, experiments were carried out to determine whether the stabilization of mRNA could be reversed when these cells are stimulated to reenter the S, G_2, and M phases of the cell cycle. It has been shown in other laboratories that the epithelial cells can be stimulated to divide when the lens is placed in organ culture. Under these conditions the epithelial cells of rabbit and rat lenses enter into synchronous division. It was felt that this technique might be applied to adult bovine lenses to determine whether the stability of mRNA in these cells could be reversed. (The stability of mRNA was determined by the ability of actinomycin D to inhibit total protein synthesis in the complete absence of RNA synthesis.) Adult lenses were placed in organ culture for various periods, removed, and incubated with ^{3}H-thymidine for 4 hr. During the first 24 hr of culture, no change in DNA synthesis occurs; after 40 hr in culture, there is a 25-fold increase in DNA synthesis; and after 64 hr there is a 60-fold increase in DNA synthesis. RNA and protein synthesis also remain at the low noncultured levels during the first 20 to 24 hr of culture and increase markedly after 40 hr of culture.

Using actinomycin D to detect the presence of stable templates during organ culture, it was found that proteins were synthesized on stable templates during the 24- and 40-hr culture periods. After 64 hr in organ culture, a significant inhibition of protein synthesis occurs. These data indicate that the stabilization of protein-synthesizing templates can be reversed in the lens epithelial cell and that this reversal may occur when these cells are induced to reenter the cell cycle.

Experiments have now been initiated to study the mechanism and chemical basis for the stabilization of mRNA. We have observed that in lens cells having stable templates there is always a decrease in protein synthesis as compared with that seen in cells where template turnover is rapid.[1,2] In view of this it was thought that stabilization of mRNA might be directly associated with the decrease in protein synthesis. Experiments were carried out to determine whether similar differences in the rate of protein synthesis seen in vivo could be shown in an in vitro protein-synthesizing system. Polysomes from adult epithelial cells and adult fiber cells were isolated and tested for their ability to support protein synthesis in vitro. It was found that polysomes from fiber cells (which contain stable templates) have a much lower incorporating capability than those from epithelial cells. When both systems were presented with polyuridylic acid as a messenger RNA, the ribosomes from fiber cells still did not attain the level of protein synthesis exhibited by epithelial cell polysomes. Single ribosomes from epithelial and fiber cell polysomal preparations were isolated by sucrose density gradient centrifugation. The single ribosomes were also tested for their ability to incorporate phenylalanine in the presence of poly U. The results were identical to those described above; that is, there is a very significant difference in the rate of incorporation of amino acids into protein in the presence of the epithelial cell and fiber cell ribosomes. These experiments demonstrate that during or immediately after lens cell differentiation the ribosomes are modified in such a manner that their efficiency in protein synthesis is decreased. The ribosomes, therefore, may contain the component or components responsible for the stabilization of mRNA. We are currently in the process of determining whether there are modifications in either the protein or RNA components of these ribosomes which might account for the decrease in protein-synthetic capacity.

References

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16.6 STUDIES ON RIBOSOMAL DEGRADATION IN LENS CELLS

W. F. Zapisek John Papaconstantinou

Electron microscopic studies of the internal structure of the lens cell have indicated that there

is a considerable loss of polysomes and ribosomes in the fiber cell.[1-4] Similar observations have been made for the reticulocyte.[5-7] Through the use of methylated albumin kieselguhr (MAK) columns, we have been able to show that there is a very marked decrease in the amount of ribosomal RNA (rRNA) extractable from fiber cells when compared with that extracted from the epithelial cells. These observations also indicate that there is a decrease in the ribosomal population of the lens fiber cell and are in agreement, therefore, with those of the electron microscopist. These experiments have been carried out with both chick and bovine lenses. Studies are being carried out to determine the mechanism of breakdown of the ribosomes.

By comparing the MAK column patterns from epithelial and fiber cells, it can be seen that there is a sharp decrease in ribosomal RNA and an "apparent" increase in the transfer RNA (tRNA). One explanation for this may be that upon degradation the rRNA may form low-molecular-weight products which have chromatographic properties on MAK columns similar to tRNA. Another explanation may be that rRNA breaks down completely, resulting in an increase in the ratio of tRNA to rRNA. Experiments were carried out to test the homogeneity of tRNA as it is prepared from MAK columns.

In one series of experiments attempts were made to separate a ^{14}C-aminoacyl-tRNA fraction from a nonchargeable soluble RNA. Lenses were incubated in the presence of a complete mixture of ^{14}C-amino acids. The RNA was extracted under acid conditions to retain the aminoacyl-tRNA linkage. This RNA was then fractionated by DEAE chromatography and by prolonged sucrose density gradient centrifugation. In both cases, the optical density at 260 mμ and the ^{14}C-labeled material formed superimposable peaks.

Further analyses were carried out with cesium sulfate equilibrium density gradient centrifugation. A uniform peak having a density of 1.68 g/cc was formed. Analysis of this RNA on acrylamide disk electrophoresis showed a single broad band. Thus by all criteria the soluble RNA from chick lens fiber cells appears to be tRNA and not contaminated by rRNA breakdown products.

Experiments have been initiated to determine whether there are rRNA breakdown products associated with another RNA fraction. In these experiments, both epithelial and fiber cells were subjected to differential centrifugation, and the supernatant and pellet fractions were treated separately by phenol extraction. The RNA extracted from these fractions was placed on acrylamide disk electrophoresis, which excludes 28s and 18s RNA. From the epithelial cell preparation it was found that the supernatant fraction contained large RNA species having a molecular weight of approximately 3 to 4×10^5. The pellet contained RNA species having a molecular weight of approximately 4 to 5×10^4. Since these particles in the pellet could be sedimented by high-speed centrifugation, it is possible that they were initially bound to protein which was stripped off by the phenol extraction.

In the fiber cells, only a very small amount of RNA (exclusive of tRNA) was found in the supernatant fraction. The pellet contained low-molecular-weight species of RNA similar to those observed in the epithelial cells. Again, it is possible that these small molecules could be sedimented because they were bound to protein. These preliminary data indicate that rRNA may be degraded in situ as RNP particles. Attempts to isolate these particles are now in progress. In addition, experiments are being carried out to further study the mechanism of breakdown of the ribosomes in the lens fiber cell.

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17. Enzymology

G. David Novelli[a]

Protein Synthesis (17.1–17.6)

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Audrey N. Best[a]
Peter Pfuderer
E. F. Phares
Lawrence Rosen[a]
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C. J. Wust
D. H. Brown[b]
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Willie M. Mitchell
Donna G. Shaffer
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Martha E. Depine[f]
D. R. Holladay[g]

Enzymatic Mechanisms in Protein Synthesis (17.7, 17.8)

M. P. Stulberg[a]
L. R. Shugart[a]
R. L. Pearson[h]
K. R. Isham
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Mammalian Cell Regulation (17.9, 17.10)

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Virginia W. Holten[c]
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D. R. Twardzik[e]

Participation in Cooperative Programs:

AEC-NIGMS (26.50–26.53)
AEC-NCI Cocarcinogenesis (26.3–26.10)

[a]Dual assignments
[b]USPHS Postdoctoral Fellow
[c]American Cancer Society Postdoctoral Fellow
[d]Visiting investigator from abroad
[e]Loanee
[f]Student trainee
[g]Temporary
[h]Chemical Technology Division, Oak Ridge National Laboratory
[i]Biology Division postdoctoral investigator

17.1 ISOACCEPTING tRNA'S IN MOUSE PLASMA CELL TUMORS

Wen-Kuang Yang G. David Novelli

In previous reports[1,2] we described the methods for preparing tRNA's and aminoacyl-tRNA synthetases from mouse plasma cell tumor tissue and demonstrated a complete profile of isoaccepting tRNA's for the 20 amino acids by using the reversed-phase column chromatography of Weiss and Kelmers.[3] The multiplicity of isoaccepting tRNA's for all the amino acids, except tryptophan, in the plasma cell tumor consisting of monoclonal cells was experimentally shown not to be due to artifacts. The biochemical and biological meaning of these isoaccepting tRNA's has been under our investigation. To examine whether isoaccepting

tRNA's may be involved in the synthesis of myeloma immunoglobulins, comparisons of various aminoacyl tRNA's from different mouse plasma cell tumors have been perfomed by cochromatography on the reversed-phase Freon column.

Results and Discussion. – *MOPC 31B and MOPC 31C.* – MOPC 31B produces unbalanced amounts of subunits L and H chains of immunoglobulin G and excretes Bence-Jones protein. MOPC 31C produces the same but complete myeloma immunoglobulin. Lysyl, leucyl, aspartyl, histidyl, and methionyl tRNA's were compared in these two tissues. Nearly identical patterns of these aminoacyl tRNA's were found except methionyl tRNA's, which showed a slight difference in the relative quantity of the three isoaccepting species detected by chromatography.

Myeloma Immunoglobulin A Producer (MPC 62)[4] *and Immunoglobulin G Producers (MPC 47 and MOPC 31C).* – Among the various aminoacyl-tRNA's analyzed, seryl-tRNA's showed the largest differences in these two kinds of plasma cell tumors. The first of the four main peaks of seryl-tRNA is prominent in IgG producers but minor in the IgA producer; while seryl-tRNA^{IV} is major in the IgA tumor but nearly absent in IgG tumors. The changes of these seryl-tRNA's were not due to (1) enzyme preparations used, (2) subcellular distribution of seryl-tRNA's, (3) lack of a pCpCpA terminus of certain of the tRNA's, or (4) modification during tRNA preparation. The four seryl-tRNA's showed no difference in their enzyme specificity, in the stability of the ester linkage with serine, or in the chromatographic location of the seryl-oligonucleotides obtained from a T_1 ribonuclease digest. Preliminary results of binding studies using various copolyribonucleotides showed that the four species of seryl-tRNA's recognized different genetic codes. These results established that plasma cell tumors with different functions of protein synthesis may have different patterns of certain isoaccepting tRNA's. With isolated polysomes and supernatant enzyme preparations devoid of tRNA, serine incorporation into protein appeared to be supported equally well by the heterologous tRNA as by the homologous tRNA from these plasma cell tumors. However, whether this difference in seryl-tRNA's is directly related to the formation of different myeloma immunoglobulins or is a response to the requirement for the translation of different genetic messages remains to be determined.

References

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[2]W. K. Yang and G. D. Novelli, *Proc. Natl. Acad. Sci. U.S.*, in press, 1968.

[3]J. F. Weiss and A. D. Kelmers, *Biochemistry* **6**, 2507 (1967).

[4]The generous supply of MPC 47 and MPC 62 mouse plasma cell tumor lines by Drs. Hooper and Albright of the Radiation Immunology group is acknowledged.

17.2 TRANSFER RNA's DURING DEVELOPMENT IN *ARTEMIA SALINA*

J. C. Bagshaw F. J. Finamore[1]
G. David Novelli

The brine shrimp, *Artemia salina*, provides a developing system amenable to biochemical studies. The encysted blastulae, which are a normal stage in the life cycle, constitute a reliable reference point, and they develop uniformly and almost synchronously to nauplii (the larval form). tRNA and crude aminoacyl tRNA synthetases have been prepared from both cysts and nauplii. Aminoacyl tRNA's labeled with ^{14}C or ^{3}H are fractionated by Freon reversed-phase chromatography, which gives good separation of different tRNA's accepting the same amino acid. Thus by cochromatography of, for example, cyst ^{14}C-leucyl-tRNA and nauplius ^{3}H-leucyl-tRNA, a direct comparison can be made of leucine tRNA's present at two different stages of development. Results to date show alterations during development in the relative proportions of the different leucine-accepting tRNA's. The same is true for arginine and alanine tRNA's. Phenylalanine and threonine tRNA's, on the other hand, appear to be identical in the cyst and the nauplius. The work is presently being extended to include other amino acids.

Reference

[1]Developmental Biochemistry section.

17.3 PROTEIN SYNTHESIS OF ISOLATED POLYSOMES

Peter Pfuderer Donna G. Shaffer

In vitro incorporation studies have failed to show a good correspondence between gross concentration of polysomal peaks and endogenous amino acid incorporation. The usual polyribosomes seen and identified in the analytical ultracentrifuge or the sucrose density gradient are not very active in endogenous (no added messenger RNA) amino acid incorporation. These *E. coli* polyribosomes have been characterized by sedimentation coefficients of 30S and 50S for the subunits; 69S for the monomer; and 102S, 129S, and 152S for the dimer, trimer, and tetramer respectively. The endogenous activity in these polysome preparations occurs at sedimentation values of approximately 85S, 120S, and 160S, where there are no discernible peaks. If the previously mentioned polysomes were true polysomes, that is, if the ribosomes were held together by messenger RNA and were not simply aggregated monomers, we would have expected activity peaks at 102S, 129S, and 152S, where the dimer, trimer, and tetramer were. The monomer peak at 69S probably would not respond in this assay if it were a ribosome end product from polysomes, as is currently believed.

A polysome of 85S has now been produced by breaking the *E. coli* at higher pressures in the French press. Under these conditions very few polysomes are produced, and the 85S particle is the predominant peak. If this particle is composed of equal numbers of 30S and 50S subunits, as has been reported in the literature, it is quite possible that both the 85S and 102S particles consist of two ribosomes, the former held together by a strand of messenger RNA and the latter a magnesium aggregate. This assumption would fit the incorporation data and also some evidence in the literature which indicates that the 102S particle is a magnesium dimer and that the 85S particle consists of equal amounts of 30S and 50S subunits. It should be mentioned that the usual polysomes which are inactive in the endogenous assay are quite active when additional messenger RNA is added to the assay mixture.

17.4 HYDROLYSIS OF OLIGORIBONUCLEOTIDES BY AN ENZYME FRACTION FROM *ESCHERICHIA COLI* [1]

Audrey L. Stevens S. K. Niyogi [2]

Certain in vivo studies suggest that messenger RNA is degraded from the 5′-termini. No ribonuclease previously described degrades RNA from the 5′-end, and none previously described from *E. coli* hydrolyzes short oligoribonucleotides to yield 5′-nucleotides. In a search for another ribonuclease that might be involved in the degradation of messenger RNA, it was found that pApA was hydrolyzed to 5′-AMP by crude extracts of *E. coli*.

An enzyme was purified tenfold from crude alumina-ground extracts of *E. coli* on the basis of its rate of hydrolysis of pApA. It rapidly hydrolyzes adenine- and uracil-containing oligoribonucleotides with a 5′-phosphomonoester end group or with no phosphomonoester end group. In all cases, 5′-nucleotides and nucleosides are the products. Its rate of hydrolysis of oligoribonucleotides with a 3′-phosphomonoester end group or of oligodeoxyribonucleotides with a 5′-end group is only 10% of the former compounds. The hydrolysis of polynucleotides has not yet been studied because of contamination of the enzyme fraction with RNase II. Further purification of the enzyme fraction is in progress. With the more purified enzyme, studies on its mode of hydrolysis will be carried out.

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[2]Nucleic Acid Enzymology section.

17.5 STUDIES OF COMPLEXES OF RNA POLYMERASE FROM *E. COLI* WITH POLYRIBONUCLEOTIDES

Audrey L. Stevens

DNA-dependent RNA polymerase from *E.* coli binds readily to DNA and polyribonucleotides. The complexes of the enzyme and polynucleotides

can be isolated by sucrose density gradient centrifugation. Using small polyribonucleotides – poly A, poly C, poly U, poly AU, and tRNA – different features of the binding reaction have been investigated.

At low ionic strength and in the presence of divalent cation, two complexes of enzyme and polyribonucleotide having sedimentation values of approximately 15s and 21s are detectable by sucrose density gradient centrifugation. The 15s complex is the main component. In the absence of divalent cation, only a 13s complex is formed. A study of the effect of increasing NH_4Cl concentrations on the sedimentation properties and on the stability of the complexes shows that a 13s complex is also formed at higher salt concentrations (0.1 *M*). The poly A–enzyme complex is most sensitive to increased ionic strength, being dissociated completely by 0.2 *M* NH_4Cl. The poly U–enzyme and tRNA–enzyme are the most stable complexes, being dissociated by 0.4 *M* NH_4Cl. Studies of competition between each of the synthetic polyribonucleotides and tRNA for enzyme suggest that the order of reactivity of the polyribonucleotides is as follows: poly A $\cong$ tRNA < poly AU $\cong$ poly C < poly U.

17.6 PILOT PLANT ACTIVITIES

Mary V. Long E. F. Phares

During the past year we ran some 73 fermentations for five groups in the Biology Division, the Macromolecular Separations Program, the Chemistry Division of ORNL, and visiting investigators from the University of Tennessee, the University of Georgia, Marquette University, Rosary Hill College, and the Ames Research Laboratory. These varied in size from 26 liters to 400 liters in continuous fermentation for the Macromolecular Separations Program. The kinds of organisms grown were: various strains of *E. coli, Clostridium pasteurianum, Aerobacter aerogenes, Photobacterium phosphoreum, Thiobacillus neopolitanus, Thiobacillus thiopasus, Desulfovibrio desulfuricans,* and *Desulfovibrio gigas.* One interesting run involved the production of 750 g (dry weight) of the fungus *Galerina marginata* for the neutron diffraction crystallography of amanita toxin by Dr. Carroll Johnson of the Chemistry Division. This was of interest since the cultural conditions for the optimum growth of this organism had not been previously determined and since it had not been grown on a large scale previously. In trial runs we worked out growth conditions that gave a good yield of toxin and finally were able to obtain good growth on a 225-liter scale.

In general it has been our experience that whenever a visiting investigator brings his own organism to be grown in our fermentor, we could work out conditions with our equipment to give from 10- to 40-fold increases in the particular product being sought. We are convinced that most investigators working with microorganisms do not take the effort to determine optimum conditions for scaling up a fermentation. We know from experience that a simple direct scale-up is not usually successful, but that all variables need to be reexamined under the new conditions.

17.7 THE INTERACTION OF PHENYLALANYL-RNA SYNTHETASE AND $tRNA^{Phe}$

M. P. Stulberg S. A. Fairfield[1]
T. W. Martin, Jr. K. R. Isham
J. W. Longworth[2]

Continued studies on the interaction of highly purified Phe-RNA synthetase and $tRNA^{Phe}$[3] have been hampered by unavailability of pure tRNA and the need for further instrumentation. Nevertheless, observed properties of substrate-enzyme binding have added to our understanding of fluorescence data concerning the interaction.

Protection of synthetase activity against heat inactivation by either ATP or tRNA has been observed. This phenomenon is usually interpreted as protection due to binding of the substrates by the enzyme. Protection by ATP is reversed by Mg^{2+} ions, and protection by $tRNA^{Phe}$ occurs in the absence of phenylalanyl-tRNA formation. Evidently nonacylated $tRNA^{Phe}$ alone can bind to the enzyme. Previous data[3] indicated that fluorescence quenching of the enzyme ($tRNA^{Phe}$-synthetase interaction) occurred only in the presence of a net reaction. The combined observations suggest that the quenching phenomenon is quite specific and due to the formation of a ternary complex of phenylalanyl adenylate, synthetase, and $tRNA^{Phe}$, accompanied by a conformational change of the protein. On the other hand, the binary complex of synthetase and $tRNA^{Phe}$ (demonstrated by protection against heat inactivation) is probably

only a partially specific binding, not detected by fluorescence quenching, and thereby without an accompanying change in protein conformation. The recent acquisition of circular dichroism instrumentation may allow us to observe the predicted conformational changes upon specific conditions of synthetase-tRNA interaction.

The almost uniquely large molecular weight of the synthetase (180,000), compared with other aminoacyl-RNA synthetases, suggested the likelihood of a subunit or polymeric protein. However, all studies on the dissociation of the enzyme by known inhibitors and classical protein-dissociating agents have not demonstrated dissociation into subunits or monomers. We have therefore tentatively assumed that a dissociation-type control mechanism does not operate in the phenylalanine activating system.

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[1]Student in The University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences.

[2]Biophysics section.

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17.8 STUDIES ON MULTIPLE $tRNA^{His}$ IN HISTIDINE-REPRESSED SYSTEMS FROM *BACILLUS SUBTILIS*

M. P. Stulberg K. R. Isham
Audrey L. Stevens

Several observations in the literature indicate an association of specific tRNA's with the initial event in repression of amino acid–biosynthetic enzymes by the corresponding amino acid. The histidine-biosynthetic enzymes in *Bacillus subtilis* are repressed when the organism is grown in the presence of histidine and derepressed when grown on histidinol (the immediate precursor to histidine). The superior resolving capability of Freon reversed-phase chromatography[1] (RPC-2) has allowed us to examine the pattern of $tRNA's^{His}$ for possible differences under conditions of repression-derepression.

Preliminary results indicate that three species of $tRNA^{His}$ are distinguishable after RPC-2 of extracted, purified tRNA. The three species differ in proportion when tRNA from repressed cells is compared with that from derepressed cells. Presently we are examining whether these differences can be associated with the repression phenomena and whether they can be attributed to interconversions of existing species of $tRNA^{His}$.

Reference

[1]J. F. Weiss and A. D. Kelmers, *Biochemistry* **6**, 2507 (1967).

17.9 ISOENZYMES: THEIR REGULATION AND GENETIC CONTROL

K. Bruce Jacobson J. A. Knopp[1]
J. B. Murphy

We have chosen to study alcohol dehydrogenase of *Drosophila* because (1) it exists in at least five electrophoretic forms[2] and (2) all these forms are under the control of a single gene.[3] Our aim is to explain the means by which these various forms of the one enzyme arise.

The alcohol dehydrogenase has been purified to a homogeneous state using standard procedures.

Let us designate the five electrophoretic forms as ADH_1, ADH_2, ADH_3, ADH_4, and ADH_5 in order of decreasing electrophoretic mobility. We have learned how to convert one form to another. For conversion of ADH_1-ADH_4 to ADH_5, the naturally occurring mixture is adsorbed to DEAE-cellulose. After several hours the enzyme consists only of ADH_5 and may be recovered in high yield (90%). For conversion of ADH_5 to ADH_3 and ADH_1, the enzyme is simply incubated with NAD or NADH; ADH_5 disappears and ADH_3 appears, then a little later ADH_1 also appears, and, finally, the only form present is ADH_1. These two experiments are consistent with the notion that the various forms of alcohol dehydrogenase result from firm binding of NAD to the protein. There are approximately four NAD per ADH in ADH_1 and <0.2 NAD per ADH in ADH_5.

The process by which NAD and NADH interact with ADH_5 is being examined. On adding NADH to ADH_5 a strong fluorescence with a maximum at 430 mμ results that is about 30 times the fluorescence of the separate components. On titrating ADH_5 with NADH, this 30-fold enhancement of

fluorescence is seen until there are equivalent molar amounts of NADH and ADH_5. Then less enhancement is seen until there are 6 NADH per ADH, and a last complex of 13 NADH per ADH accounts for a lesser enhancement yet. After that point no enhancement occurs on adding additional NADH. These complexes that form are reversible and thus are not able in themselves to explain the conversion of ADH_5 to ADH_1 because the enzyme retains the mobility characteristic of ADH_5. However, they may prove to be the first step in the conversion process, and we intend to continue the application of fluorescence technics in this study.

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17.10 *E. COLI* VALINE tRNA SYNTHETASE: PURIFICATION

P. O. Ritter K. Bruce Jacobson

It has been reported that both a phenylalanine tRNA synthetase from *Neurospora crassa* and a valine tRNA synthetase from *E. coli* can catalyze the charging of $tRNA^{Val}$ (*E. coli*).[1] The *Neurospora* enzyme catalyzes the transfer of phenylalanine to the $tRNA^{Val}$ (*E. coli*).[2] The purified enzymes, together with purified $tRNA^{Val}$, will be used to study the synthetase recognition site(s) on the tRNA molecule.[3] Initial studies will involve an examination of the effects of various modifications of the tRNA molecule on the kinetics of the two enzyme reactions.

The *E. coli* valine tRNA synthetase has been partially purified with the use of the following procedure. *E. coli* B cells are passed through a Gaulin press, and the resultant mixture is centrifuged. Most of the nucleic acids are removed from the supernatant with streptomycin sulfate; then a 50 to 65% ammonium sulfate cut is collected. The enzyme solution obtained from the ammonium sulfate fractionation is heated in the presence of ATP, valine, and Mg^{2+}, and the nonprecipitated protein is passed through a DEAE-cellulose column. The enzyme is further purified by chromatography on a hydroxylapatite column followed by rechromatography on a DEAE-cellulose column. Cellulose acetate membrane electrophoresis at pH 8.5 indicates that the enzyme removed from the second DEAE-cellulose column contains only one minor contaminant (estimated to represent less than 10% of the total protein). Ammonium sulfate fractionation or further chromatography will be used in an attempt to obtain a homogeneous enzyme preparation.

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18. Nucleic Acid Chemistry

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18.1 tRNA STRUCTURE AND FUNCTION

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Because of the biological function of tRNA, one is not surprised to observe structural elements common to all tRNA's and independent of biological source, as for example the unique compositional units GTΨCG,[1] CCA,[2] and the C · G initiating base pair.[3] The observations of marked compositional differences among tRNA preparations, for example, the number and distribution of methylated bases in yeast and *E. coli*, suggest special roles for these compounds, but such have not yet become clear.

We have been investigating the sequence of $tRNA^{Phe}$ from *E. coli* B (Molecular Separations Program preparations 9-200-V, 13-104, 13-105, 13-41, and 13-83). To permit facile comparison of the various preparations with only small amounts of the tRNA at hand, we have developed highly reproducible mapping techniques. The complete hydrolysates may be analyzed by quantitative anion exchange chromatography in conjunction with quantitative nucleoside analysis,[4] so that both the position and yield of each oligonucleotide may be accurately determined. We have isolated 27 oligonucleotides in greater than 85% yield by chromatographic separation after exhaustive hydrolysis with either pancreatic RNase A (E.C. 2.7.7.16) or RNase T_1 (E.C. 2.7.7.26). In addition to the 27 major fragments, a number of oligonucleotides have been isolated in lower, variable yields depending upon the preparation and extent of activity loss during storage.

Because there are relatively few rare bases that can serve as positional markers in $tRNA^{Phe}$ (pseudouridine, ribothymidine, 4-thiouridine, dihydrouridine, and 7-methylguanosine are present), the reconstruction of the full sequence is complicated. Hence, we have begun to degrade the tRNA in sequential fashion, beginning at the adenosine terminus.[5] We have been able to carry this procedure through 15 cycles on preparation 13-105 (50% active $tRNA^{Phe}$) in order to obtain the sequence –CGAGUCCGGGCACCA. This sequence is completely consistent with an overlap of five of the oligonucleotides previously isolated in greater than 85% yield. We are continuing the sequential degradation with a more highly purified sample of $tRNA^{Phe}$.

Two unusual structural features of our $tRNA^{Phe}$ have emerged and may be compared with the published sequence for $tRNA^{Phe}$ (yeast), even though

our entire sequence is not completed. First, the virtual absence of the common *N*-methylated bases from the oligonucleotides isolated from *E. coli* $tRNA^{Phe}$ is in clear contrast to the composition of the several yeast tRNA's (including yeast $tRNA^{Phe}$) and rules out any special universal role for *N*-methylated bases in tRNA function. However, the presence of 7-methylguanosine in $tRNA^{Phe}$ from both sources and its absence from the other yeast tRNA's so far sequenced suggests that this group may be involved in a function peculiar to all $tRNA^{Phe}$'s.

Second, and probably of broader significance, is the following observation: The sequence –GAAAΨCCCCG– is the only oligonucleotide isolated that contains acceptable anticodons for phenylalanine. This sequence contains the two anticodons permitted by the wobble hypothesis, arranged in overlapping fashion (GAAA). It is not possible at this time to eliminate the AAA sequence as a contributor to the anticodon function. This gives rise to the speculation that either of the anticodon triplets may be used (one at a time) by a simple conformational shift in the messenger RNA or the tRNA, so as to permit the codon to associate with AAA instead of GAA.

Such a conformational shift leading to utilization of the other anticodon appears sterically possible. Fuller and Hodgson,[6] from the yeast tRNA data,[1] have constructed a model for the anticodon loop and conclude that three overlapping triplet positions (a quintet) in the anticodon region are accessible to the messenger codon, two of these having a much higher probability of interaction in the tRNA-messenger-ribosome complex. The two probable sets are in the same relative position as the GAAA sequence in our $tRNA^{Phe}$ (*E. coli*), thus giving more weight to the thought that either anticodon may be used.

An analysis of the trinucleotide-dependent binding of $tRNA^{Ser}$ (yeast)[7] to ribosomes indicates a possible extension of this concept. There is an anomalous dependence upon AGU and AGC trinucleotides that may arise from the utilization of a triplet other than the accepted anticodon triplet. Examination of the $tRNA^{Ser}$ structures (there are two) reveals the triplets UCU and UUU located in a loop supported by a five-base-pair helical region, strategically located between the GTΨCG and H_2U region very close to the normal position for the accepted anticodon region. A conformational shift similar to that described above may allow trinucleotides to associate with the triplets in this loop and thus bring about the binding of aminoacyl-tRNA to ribosomes that is actually observed.

At present this speculation can be entertained only for the trinucleotide-dependent binding experiments and may not occur when all components for protein synthesis, including a bona fide messenger RNA, are present. However, there are as yet no grounds for excluding the trinucleotide-dependent binding results as abnormal or artifactual.

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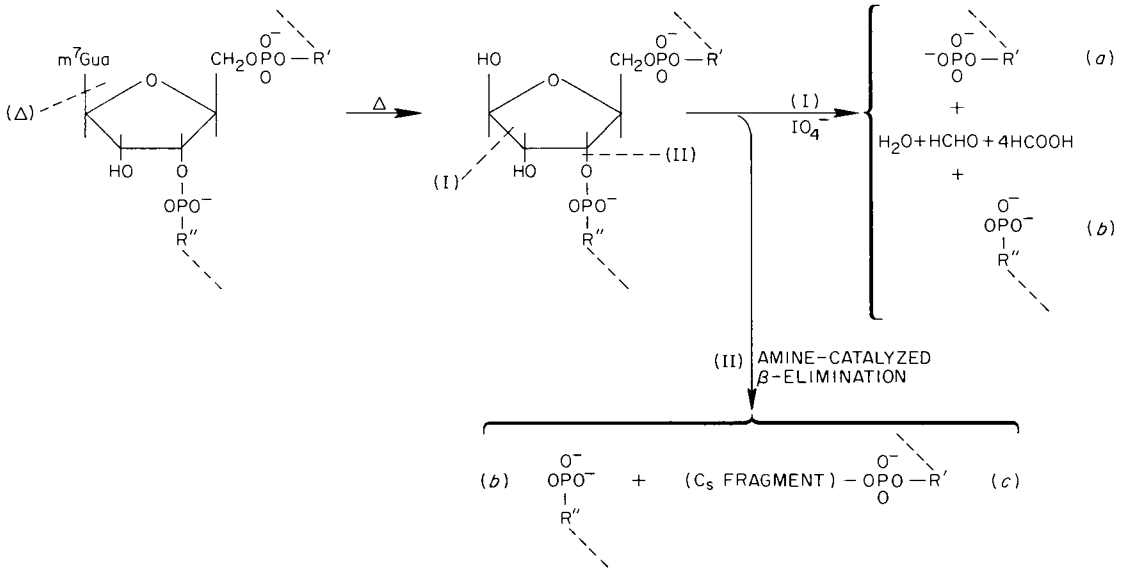
18.2 A SPECIFIC CLEAVAGE OF tRNA CHAINS

Mayo Uziel

A recurring problem in nucleic acid structure determination is the preparation of large fragments for the final stage of sequence reconstruction. The ideal way to cleave the chain selectively is to exploit a single sensitive residue. Such a residue is present in $tRNA^{Phe}$ and can be treated in a highly selective fashion.

7-Methylguanosine may be depurinated at neutral pH by heating in an aqueous solution (see step Δ in diagram below). The resulting polymer can then be separated into two fragments by either β elimination or oxidative hydrolysis of the phosphate ester. Quantitative analysis of the end groups (fragment *a*) formed after procedure I (periodate oxidation) shows an equivalence to the amount of 7-methylguanine released by heat. Over 90% of the 7-methylguanosine either in the RNA chain or as the free nucleoside is depurinated. Chromatography of the final products (*a* and *b*) indicates that fragmentation of the *E. coli* tRNA chains does occur.



18.3 ON THE USE OF TLC AND TLE IN THE SEPARATION AND CHARACTERIZATION OF OLIGONUCLEOTIDES

H. G. Gassen

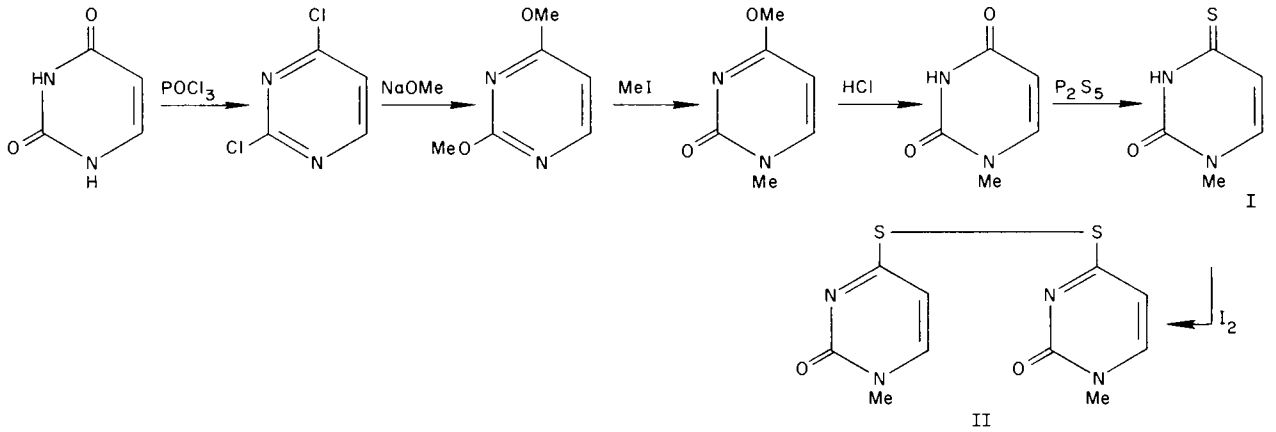
We have developed thin-layer electrophoresis (TLE) and thin-layer chromatography (TLC) into a sensitive and rapid method for the separation of the oligonucleotides obtained from enzymatic hydrolysates of RNA. TLE alone or combined in a two-dimensional system with TLC was applied in two different ways: (1) Oligonucleotide mixtures, obtained from column separation or from enzymatic digests of larger oligonucleotides, were resolved by TLE, thus avoiding a second column separation. (2) Enzymatic hydrolysates of RNA were mapped using TLE followed by TLC. TLE was performed in a locally constructed Markham-Smith electrophoresis apparatus adapted for precoated plastic foils, whereas TLC utilized commercially available equipment. As little as 2 to 5 nanomoles of material may be applied to the layer and detected after separation. A typical separation uses 1000 to 1300 v for 30 to 45 min in 0.1 *M* $HCOONH_4$/HCOOH, pH 2.45, although other pH's may be used. If the mixture is too complex, it may be followed by chromatography in the second dimension on the same plate in *t*-butanol–formate buffer or another solvent.

The substances located by uv were eluted with 0.2 ml H_2O and analyzed, after hydrolysis to the nucleoside level, with the nucleoside analyzer. The recovery drops from ~95% for the mono- and dinucleotides to about 70% for penta- and hexanucleotides. Longer oligonucleotides cannot be resolved by this technique. However, the method has a number of advantages, especially its 10- to 20-fold increased sensitivity compared with paper. The spots are distinct, about 0.5 cm in diameter, and about 0.01 ODU can be detected. They can be eluted with 0.2 ml H_2O, and the solutions are nearly salt free. The Re and Rf values are helpful in the identification of the oligonucleotides. If the size of an oligonucleotide is known, its charge at given pH can be calculated from the Re value.

18.4 THE PROPERTIES OF PYRIMIDINE DISULFIDES

B. C. Pal

The recent discovery of 4-thiouridylic acid in the soluble *E. coli* RNA[1] indicates the possible existence of –S–S– bonds in nucleic acids.[2,3] A study of the properties of the disulfide of 4-thiouridylate is essential if the existence of the disulfide linkage in nucleic acids is to be demonstrated and its behavior understood. With this object in mind, we have synthesized the methyl analog (II) of 4-thiouridine disulfide as a model compound by the method of Fox *et al.*[4]



The compound **II** crystallizes slowly from cold water as long needles (mp 227° d.). An x-ray crystallographic study of these crystals has been undertaken by Dr. Ralph Einstein. Analysis calculated for $C_{10}H_{10}O_2N_4S_2$: C, 42.54; H, 3.57; N, 19.85; S, 22.71. Found: C, 42.57; H, 3.55; N, 20.04; S, 22.90.

As expected, the uv absorption spectra of **II** at pH's 2, 7, and 12 are similar to those of 4-thiouridine disulfide. Both the compounds have been found to be unstable in alkali, liberating the 4-thio monomer (**I**) and an unidentified compound, as reported previously for the natural (1-ribosyl) derivative by Uziel[2] and Lipsett.[3] From this observation, it appears that the alkaline cleavage to yield an unidentified product is common to both the natural material and our methyl analog (**II**) and thus does not directly involve the ribosyl moieties of the former. Preliminary experiments indicate that our unknown compound hydrolyzes in dilute acid to 1-methyluracil and 1-methyl-4-thiouracil (**I**). The isolation and characterization of the unknown compound and a study of other properties of the – S – S – bond in 4-thiouracil disulfides are in progress.

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18.5 STRUCTURAL SIMILARITIES IN TRANSFER RNA's

Mayo Uziel
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Transfer RNA's are cofactors for the activation and transfer of amino acids to peptide linkages in protein synthesis. Because of their common function, they should contain common structural elements, and these should be reflected in sequence similarities.

Of the various tRNA molecules of known sequence, several such features are evident from the base distribution, one of which is the initiation of the terminal double-helical region by a base pair at position 5 from the adenosine terminus. Other features are the presence of 75 moles of – T – Ψ – C – G – for each 100 moles of mixed tRNA from both *E. coli* B and yeast[1] and of a terminal pG (90 per 100, with pA, pU, and pC in the remaining 10).[2]

Using stepwise degradation,[3] we have found that about 65% of the total number of chains in commercial tRNA or 80% of the tRNA chains of *E. coli* B tRNA contain cytidine in position 5 (Table 18.5.1). This is in good agreement with both the amount of terminal pG and observations by others[4] on the pyrimidine content of the fifth position of tRNA. Similar data have been obtained for yeast tRNA. Thus GC is the usual initiating base pair in tRNA.

The finding of C at position 5 in such large amounts provides additional evidence for a tRNA structural property largely (80%) independent of the biological source and of the particular amino acid specificity of a tRNA molecule.

Table 18.5.1. Sequential Degradation of *E. coli* tRNA with IO_4^- and Amine-Catalyzed Base Elimination[a]

Cycle	U	G	A	C	Total Base[b]	Total P_i[b]	Percent C[a,c]
1	5	*d*	*81*	*d*	86		
2	2	*d*	*d*	*85*	87	170	
3	*d*	7	16	*64*	87	85	94
4	5	20	40	21	86	88	33
5	2	15	15	*53*	85	90	80
6	3	23	23	37	86	84	50

[a]86 nanomoles of RNA were used for this experiment (terminal adenosine content; see c below for tRNA content).

[b]Corrected for mechanical losses.

[c]Using Zamir's data on TΨCG content[1] and those of Preiss and Berg on CCA content[5] for mixed *E. coli* tRNA, there should be about 68 nanomoles of tRNA in this preparation.

[d]Quantity was too low to measure.

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18.6 PURIFICATION OF tRNA

B. C. Pal

Liquid-Liquid Extraction of Phe-tRNA^{Phe}. – The effect of variation of salt concentration in the aqueous phase on the distribution coefficients of tRNA and Phe-tRNA^{Phe}, using an organic phase of 0.5 *M* Adogen 464 in Freon-214 and ^{14}C-labeled Phe-tRNA, has been studied. The K_D values have been calculated on the basis of OD_{260} values (for the tRNA), ignoring the contribution of Phe-tRNA. The results indicate that Phe-tRNA^{Phe} can be enriched eightfold by a single extraction in this system using the aqueous phase 0.26 *M* NaCl, 0.01 *M* acetate buffer, pH 4.6, and 0.01 *M* $MgCl_2$. In the absence of measurements of uncharged tRNA^{Phe}, no statement can be made as to the influence of the attached Phe residue on the extractability of this species. From similar although not identical results obtained by others (at X-10) on tRNA^{Phe}, it appears that the charged and uncharged species have similar extraction coefficients.

19. Nucleic Acid Enzymology

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19.1 BIOCHEMICAL ANALYSIS OF NATURALLY REJOINED SECTIONS OF DNA STRANDS OF BACTERIOPHAGE T5

R. K. Fujimura Elliot Volkin

There are at least two ways in which nucleotide incorporation into DNA can occur. One is sequential incorporation from one end during the replication process, and the other is end-to-end joining of preexisting polynucleotides. The present study is on the molecules formed by the second process, the process very likely involved in recombination and repair processes of DNA. The evidence in the literature for the involvement of a short stretch of newly incorporated oligonucleotide in such a process is indirect. The present work was initiated to get direct evidence that the formation of such oligonucleotides is involved in the rejoining process.

The experiments showed that there is formation of phosphodiester bonds between preexisting DNA strands and newly incorporated nucleotides in the absence of DNA replication. The system used was the bacteriophage DNA in T5-infected *E. coli*. In order to ensure that the nucleotides are not incorporated into newly replicated DNA, a temperature-sensitive DNA polymerase mutant T5ts53 was used at a high temperature. Furthermore, bacteria were grown in a medium containing bromodeoxyuridine so that newly replicated T5 DNA, as well as *E. coli* DNA, are denser than infecting parental T5 DNA.

The experiments and results were briefly as follows: *E. coli* F growing in bromodeoxyuridine-containing medium was transferred to a 43°C bath just before infection and infected with purified ^{32}P-labeled T5ts53 at moiety 5. Bacteria were harvested 30 min after infection. DNA was extracted by a method using lysozyme, pronase, SDS, and hot phenol. Usually 80 to 100% of phage DNA was extracted by this method as acid-insoluble fraction. Sucrose density gradient analysis of extracted DNA showed some fragmentation. How-

ever, the degree of fragmentation differs from experiment to experiment, and it did not seem to have an effect on the final result. Thus, it was caused most likely during the extraction.

The extracted DNA was purified by CsCl density gradient centrifugation, followed by alkaline digestion. The sample was then divided into two fractions and hydrolyzed enzymically, one to 5′ nucleotides and another to 3′ nucleotides. From each fraction, bromodeoxyuridylic acid was isolated in the presence of the corresponding ^{3}H-dBrUMP as markers by means of Dowex 1 Cl^{-} chromatography. The ^{32}P-labeled dBrUMP should form only at the joints; thus, it was small in quantity with respect to other nucleotides and could not be detected just by Dowex 1 chromatography. The samples under the ^{3}H-dBrUMP region were pooled, concentrated, and further purified by two-step paper electrophoresis – first at pH 2.7 and then at pH 9.3. The results after the electrophoresis in pH 9.3 are shown in Fig. 19.1.1. As can be seen, there was no ^{32}P activity under ^{3}H-5′-dBrUMP. However, ^{32}P activity that is almost exactly superimposable with ^{3}H-3′-dBrUMP was present. The identity of the peaks was further confirmed by treatment of the samples from Dowex 1 with venom 5′ nucleotidase and alkaline phosphatase. The 5′ nucleotidase degraded ^{3}H-5′-dBrUMP to nucleosides but did not attack ^{3}H-3′-dBrUMP or the ^{32}P activity superimposed on it. However, alkaline phosphatase did degrade both ^{3}H- and ^{32}P-labeled substances. Thus, they could only be 3′-dBrUMP. The identity of the slower moving ^{32}P peak is unknown, but it is not dUMP and is not sensitive to alkaline phosphatase.

These results are consistent with the following model of end-to-end joining of the preexisting strands.

1. The DNA strands are broken, leaving P at the 5′ position of deoxyribose.

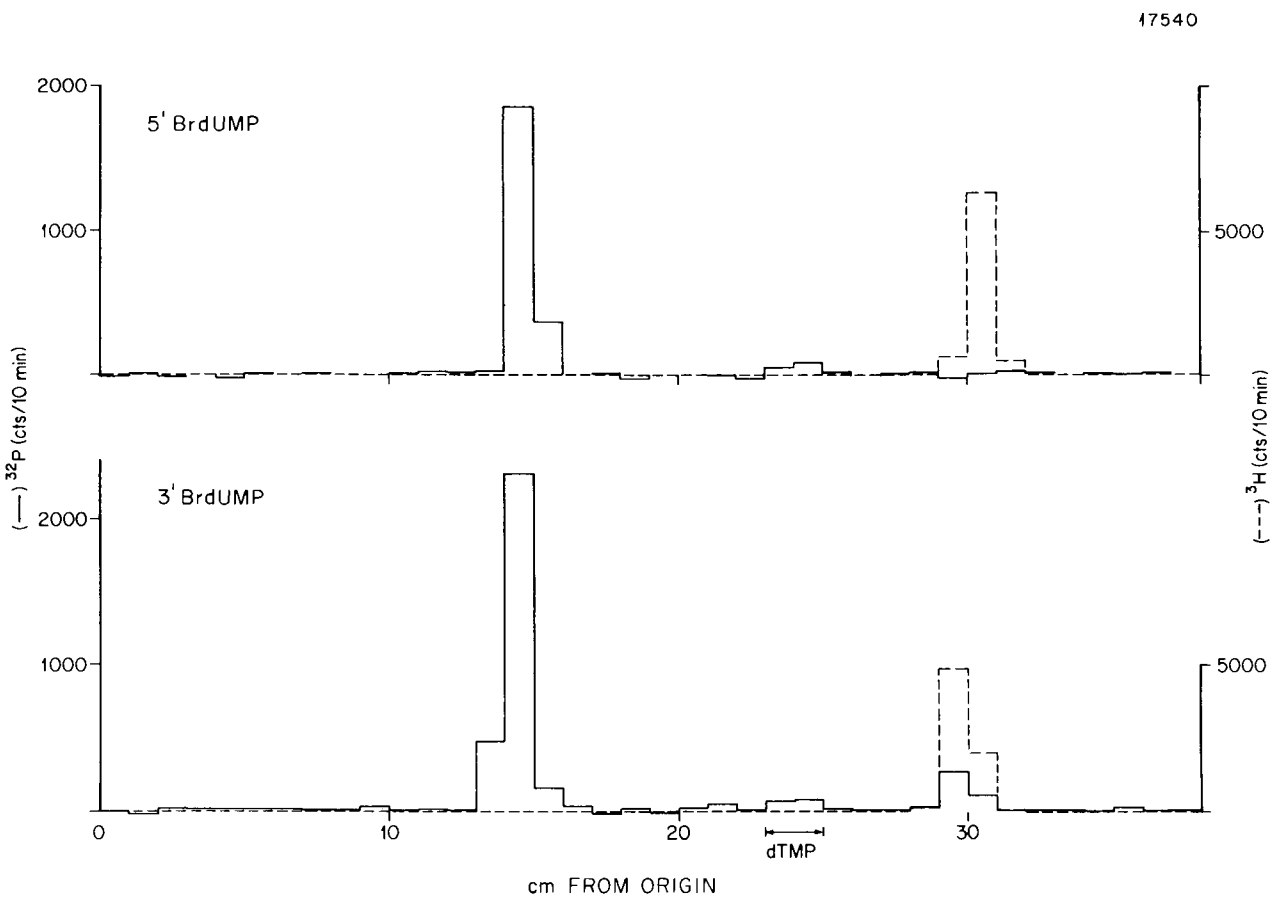

Fig. 19.1.1. Electrophoresis of BrdUMP's.

2. New 5′ nucleotides are incorporated into these gaps, joining two preexisting DNA strands.

If such a rejoined DNA is digested to 5′ and 3′ nucleotides as was done in the present work, ^{32}P label from a polynucleotide is transferred only to 3′ position of newly incorporated nucleotides; thus only 3′-dBrUMP will be ^{32}P labeled. On the basis of the fraction of ^{32}P obtained as 3′-dBrUMP, it can be calculated that the number of such nucleotides is about 25 per molecule. Assuming 50% of T is substituted by dBrUr, the number of joints per molecule is about 200. Thus this is too large a number to be just a repair of preexisting natural nicks in T5 DNA.

There may be two other possible explanations:

1. It could be degradation and reincorporation of nucleotides from parental DNA. This is unlikely because there was no extensive degradation, and the result was independent of degree of the fragmentation of DNA extracted. Also, there is no DNA replication in control experiments until about 90 min after the infection, even at 30°C.
2. It could be by the replication of DNA near the initiation site, and such a site may be covalently linked to parental DNA. This is unlikely because there are too many joints per molecule to be at initiating point(s) of replication.

However, the reverse experiment, that is, dBrUr-phage infection of bacteria in ^{32}P medium, is in progress, and the ^{32}P incorporation into joint sections is being analyzed.

19.2 PURIFICATION OF BACTERIOPHAGE T2 mRNA

W. S. Riggsby Elliot Volkin

RNA isolated from a culture of *E. coli* infected with T2 phage and grown in the presence of $^{32}PO_4$ and tritiated ribonucleosides was fractionated on a nitrocellulose (NC) column. The column contained 15 mg of T2 DNA bound to 10 g of prepared nitrocellulose powder; RNA was incubated with a slurry of powder at 66°C for 18 hr, then placed on a jacketed column at 60°C. After collection of the effluent and washing of the column with 6XSSC to remove unbound RNA, the hybridized RNA was eluted with water. In a typical experiment, 5.7 mg of RNA with a specific activity of 2.3×10^3 counts min^{-1} μg^{-1} was incubated with the DNA-NC complex. The effluent, containing 4.3 mg, had a specific activity of 8.0×10^2 counts min^{-1} μg^{-1}, while the first water fraction contained 0.24 mg and had a specific activity of 2.2×10^4 counts min^{-1} μg^{-1}. The 6XSSC fractions and the subsequent water fractions had specific activities similar to the respective fractions given above. By incubating and elution of several portions of RNA on the same column, we have prepared approximately 1 mg of the heavily labeled RNA. While this preparation may contain other types of RNA, its ability to hybridize with T2 DNA suggests that it is principally T2 messenger RNA. We are now attempting to characterize the primary structure of the purified mRNA(s).

19.3 COMPETITION IN DNA-RNA HYBRIDIZATION EXPERIMENTS

W. S. Riggsby Virginia Merriam[1]

Unlabeled RNA competes with labeled RNA for binding sites on homologous DNA immobilized on nitrocellulose disks. In principle, these experiments can be carried out in two ways: (1) simultaneous incubation, in which the labeled RNA and competing unlabeled RNA are added to the DNA-loaded disks simultaneously; and (2) preincubation, in which the disks are first exposed to unlabeled RNA, washed, and then exposed to labeled RNA.

Formal analysis of the kinetics of the hybridization reaction leads to the prediction that method 2 should be the preferred method. It has been used successfully for hybridization of viral RNA[2,3] but was reported to have failed in a mammalian system.[4]

We investigated this question in both mammalian and phage systems. In the mammalian system, we observed competition by means of the preincubation technique when we used a large concentration of labeled RNA with respect to DNA (100 to 1). In agreement with the literature, we did not observe competition when we used an RNA concentration far below the saturation level (2 to 1). In the phage system, we observed competition at all the RNA concentrations used; in these experiments the ratio of labeled RNA to DNA ranged from 0.044 to 92.5.

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19.4 THE STABILITY OF OLIGOADENYLATE-POLYURIDYLATE COMPLEXES AS MEASURED BY THERMAL CHROMATOGRAPHY[1]

S. K. Niyogi

At the present time there are two convenient ways to test the stability of polynucleotide complexes: (1) stability to ribonuclease, a procedure useful for ribonucleotides; and (2) stability to increased temperature ("melting"). The first procedure can be used with tracer levels of labeled ribonucleotides, whereas the second, which involves measuring changes in absorbance vs temperature, requires large amounts of polynucleotide. A simple method has been worked out, based on thermal chromatography, of measuring the "melting" temperature of tracer amounts of polynucleotide complexes.

The experiments involve labeled oligo A's containing either a terminal or no terminal phosphate. These compounds are allowed to interact with poly U of much greater length. Each complex of oligo A–poly U was retained on a small hydroxylapatite column that retains the poly U at all temperatures of the melting experiment but can retain the oligo A only if it is complexed with the poly U. As the temperature is gradually raised, each oligo A– poly U complex "melts," whereupon the labeled oligo A is released from the column and assayed. When the amount of oligo A eluted per degree centrigrade is plotted against temperature, a sharp peak, similar to a chromatographic peak, is seen centered around the melting temperature, *Tm*. The temperature of release (*Tm*) of the oligonucleotide is an indication of the stability of the oligo A–poly U complex. The *Tm* is directly proportional to the chain length of the oligo A and is increased in the presence of Mg^{2+} ion. An oligo A with a 5′-phosphate end group forms the most stable complex with poly U; removal of the 5′-phosphate lowers its melting temperature. The presence of a 2′- or 3′-phosphate terminal group reduces the stability even further, especially for $(Ap)_2$ and $(Ap)_3$. It is interesting that these findings are in agreement with those of McLaughlin *et al.*[2] and Rottman and Nirenberg[3] on the binding of oligonucleotides to polynucleotides on ribosomes but are in contrast with the results of Lipsett *et al.*[4] on binding to polynucleotides in solution. Perhaps the hydroxylapatite crystal surface mimics the ribosome by providing a similar pattern of interacting groups that stabilize the polynucleotide duplex. Like the ribosomes,[2] the hydroxylapatite has a stabilizing effect on these complexes, as indicated by higher *Tm*'s than in solution.

When the reciprocal of the melting temperature, $1/Tm$, is plotted against $1/N$ (the reciprocal of the oligo A chain length), the points can be fitted by a straight line extrapolating to a point on the ordinate that corresponds to about 77° at "infinite" chain length in the presence of Mg^{2+} and to about 63–66° (depending on the presence or position of the terminal phosphate) in the absence of Mg^{2+}.

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19.5 STUDIES ON THE ROLE OF CHAIN LENGTH AND BASE COMPOSITION IN THE SPECIFIC ASSOCIATION OF RIBOOLIGONUCLEOTIDES WITH DENATURED DNA

S. K. Niyogi

RNA made in vitro on a DNA (T2, T4, T3, or T7) template is degraded with nucleases and fractionated according to chain length. Previous studies[1] from this laboratory have shown that a chain length of ten or more ribonucleotides is required to form a ribonuclease-resistant com-

plex with denatured DNA, even under optimum conditions. The shortest oligomers capable of complexing with denatured DNA do so with a high degree of species specificity. For example, T7-produced oligonucleotides complex with T7 and T3 DNA but not with T2 or T4 DNA. Similarly, T2-produced oligonucleotides complex with T2 and T4 but not with T3 or T7 DNA. Homology is known to be greater between two "odds" or two "evens" than between an "odd" and an "even" phage DNA.

The ratio (A + U)/(G + C) of T3 and T7 oligomers (8-mers to 16-mers) ranged from 0.89 to 1.13, whereas that of T2 and T4 oligomers ranged from 1.80 to 1.94. The annealing of these oligomers was studied at different temperatures. In both cases (T odd and T even) a detectable complex can be seen with 10-mers with a three- to fourfold improvement with 11-mers. As expected, the temperature for optimum complex formation is directly proportional to the chain length of the oligonucleotides for both the T-odd and T-even cases. However, the optimum temperatures for the T-odd oligomers are considerably higher than those for the T-even ones of the same size. This is evidently a reflection of the higher G + C contents of the T-odd oligomers. Furthermore, in the case of the T-odd oligomers, upon passing from 11-mers to 12-mers there is a great enhancement of complex formation. A similar shift in the extent of complex formation is observed with the T-even oligomers; however, this occurs in passing from 12-mers to 13- and 14-mers. This shift in stability at a higher chain length for the T-even oligomers is probably due to the lower G + C content of these oligomers as compared with the T-odd ones.

The stability of homologous oligoribonucleotide–denatured DNA complexes on nitrocellulose filters has been studied by "thermal chromatography."[2] The "melting" temperature increases with the chain length of the oligonucleotide, and T3 and T7 oligonucleotides melt at temperatures higher than T2 and T4 oligonucleotides of the same chain lengths. This is evidently due to the difference in base composition; the T-odd oligomers have a substantially higher G + C content than the T-even oligomers.

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19.6 THE GENETIC TRANSLATIONAL APPARATUS OF MITOCHONDRIA

W. E. Barnett
L. S. Dure III[1]
J. L. Epler
Diane J. Goins

Transfer RNA's, aminoacyl-RNA synthetases, and ribosomal RNA's, macromolecules known to be involved in genetic translation, have been isolated from *Neurospora* mitochondria and characterized. The following observations have been made:

1. Mitochondria contain a complete set of tRNA's, and the three mitochondrial tRNA's which have been examined in detail (those for leucine, alanine, and phenylalanine) are uniquely associated with the mitochondria and distinct from their counterparts found in the cytoplasm.
2. Mitochondria contain a full complement of aminoacyl-RNA synthetases, and those examined closely were found to be different from the cytoplasmic synthetases in chromatographic properties, in tRNA specificity in acylation, and in being associated exclusively with the mitochondria.
3. Ribosomal RNA's from both *Neurospora* mitochondria and cytoplasm have been characterized by sucrose density gradient centrifugation. The S values for cytoplasmic rRNA's were calculated to be 16 and 25.6, whereas the light and heavy rRNA components isolated from mitochondria were 16 and 23 S respectively. Thus the sedimentation behavior of mitochondrial rRNA's is different from that of cytoplasmic rRNA's but similar to that of rRNA's of bacterial origin.

The concept which arises from these observations (and reports from other laboratories) is that the complete sequence of transcriptional and translational events occurs within mitochondria and that the macromolecules involved are unique to this organelle and distinct from those found in and used by the cytoplasm.

Reference

[1]Department of Biochemistry, University of Georgia, Athens.

19.7 CODING PROPERTIES OF *NEUROSPORA* MITOCHONDRIAL AND CYTOPLASMIC TRANSFER RNA's

J. L. Epler W. E. Barnett
Diane J. Goins R. A. Dolbeer

Fractionation of whole-cell *Neurospora* tRNA by countercurrent distribution has resolved multiple leucine tRNA's. Similarly, *Escherichia coli* contains multiple acceptors for leucine.

In the bacterial system, observations on code word recognition of leucyl-tRNA's have pointed to the occurrence of two types: (1) tRNA's coded by CUX, where the 3′ position X may be A, G, C, or U, and (2) a tRNA coded by UUX, where X may be G or A. Another of the leucyl-tRNA's of *E. coli* responds, ambiguously, to poly U, especially at high magnesium levels. In *Neurospora*, separate leucyl-tRNA's respond preferentially to polymers containing the above codons.

We have recently shown that *Neurospora* contains tRNA's and synthetases uniquely associated with the mitochondria as well as those found in the cytoplasm. Utilizing these mitochondrial and cytoplasmic tRNA's and enzymes, we have observed that mitochondrial leucyl-tRNA responds only to UC-containing polymers in ribosomal binding, while cytoplasmic leucyl-tRNA's respond to UC-, UG-, and (ambiguously) to U-containing polymers.

Countercurrent distribution of mitochondrial tRNA has resolved the leucine species as a single peak partitioning at the position of leucine II of whole-cell tRNA. Similarly, the mitochondrial phenylalanine tRNA corresponds only to phenylalanine I of whole-cell tRNA and is specifically charged by the mitochondrial synthetase. Both phenylalanine I and II show a coding to poly U and U-containing polymers.

A detailed examination of the coding properties of mitochondrial tRNA's is in progress.

19.8 DNA SYNTHESIS IN *ARTEMIA SALINA*

C. G. Mead

The enzymatic mechanism responsible for the in vivo synthesis of DNA in *Artemia salina* has not been demonstrated in vitro. The classical type of DNA polymerase is not demonstrable in extracts of nauplii even at times when DNA synthesis is occurring at a maximum rate in vivo. There appears to be no inhibitor present in these extracts, since *E. coli* DNA polymerase is unaffected when added to the extracts.

The possibility is being examined that the enzyme responsible for DNA synthesis in *Artemia salina* utilizes substrates other than the normal deoxynucleoside triphosphates. Finamore has shown a direct relationship in vivo between the utilization of P^1,P^4-diguanosine 5′-tetraphosphate and the increase in DNA adenine. A highly labeled compound has been isolated from acid-soluble extracts of nauplii grown in seawater supplemented with $^{32}PO_4$ that has some properties expected of an intermediate in the conversion of P^1,P^4-diguanosine 5′-tetraphosphate to an adenine derivative. Its chromatographic behavior on both DEAE and Dowex 1 Cl^- columns is that expected of a dinucleoside tetraphosphate. The absorption spectrum of this compound is not characteristic of either a guanine-containing compound or an adenine-containing compound but is intermediate. Identification of this compound is currently in progress.

19.9 NUCLEOTIDE SEQUENCE ANALYSES

J. X. Khym Mayo Uziel[1]

Recently we have shown that the cetyltrimethylammonium (CTA) cation can be used effectively (as a precipitant for polyribonucleotides) to ease the manipulations involved in the cyclic stepwise degradation procedure that was designed to determine the sequence of nucleotides in RNA. Details are given elsewhere.[2,3]

The -CpCpA terminus of tRNA (*E. coli*) was repeatedly determined to check the reproducibility of the CTA method. The amounts of base found at each cycle in these experiments are shown in Table 19.9.1. Once the reliability of the method had been established, it was used in sequence studies of tRNA from both *E. coli* and yeast (see report given in the Nucleic Acid Chemistry group).

The lower limit in terms of the size of oligonucleotides that are quantitatively precipitated by CTA is apparently a polymer that is approximately 15 nucleotides in length. This was determined by solubility studies of CTA in the presence of oli-

Table 19.9.1. Precision of Sequence Method in Determining the -CpCpA Terminus of tRNA[a]

Cycle	Base	Nanomoles[b]	Range
1	A	890	870–920
2	C	910	820–920
3	C	900	870–930

[a]About 930 absorbancy units of tRNA were used in each single determination. Alkaline hydrolysis of starting material yielded 930 nanomoles of adenosine per 930 absorbancy units taken for analysis.

[b]Numbers represent average nanomoles found for six determinations.

gonucleotides of varying length that were isolated from Takadiastase RNase digests of ribosomal RNA (rRNA). The solubility, in the presence of CTA, of the higher molecular weight polymers such as rRNA and TMV is also being investigated.

Initial sequence studies were carried out on a 1-micromole scale (i.e., the release of 1 micromole of terminal base per cycle). Later experiments showed that sequences of polyribonucleotides could be determined conveniently at the 0.1-micromole level. Studies are now in progress to determine whether the CTA procedure can be used for sequence work in which 0.01 micromole of base is released in each cycle of the degradation procedure.

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20. Developmental Biochemistry

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20.1 ROLE OF P^1,P^4-DIGUANOSINE 5′-TETRAPHOSPHATE IN THE SYNTHESIS OF DNA BY *ARTEMIA SALINA*

F. J. Finamore Rose P. Feldman

The nucleotide pool of newly hatched nauplii of the brine shrimp *Artemia salina* contains large quantities of guanine derivatives, particularly diguanosine tetraphosphate, but only small amounts of adenine compounds. These larvae are unable to synthesize purines *de novo*, and as a consequence they convert guanine to adenine as it is required by the organism. Furthermore we recently reported[1] that diguanosine tetraphosphate most probably serves as the major source of the adenine derivatives.

In an effort to gain some insight into the mechanism by which guanine derivatives are converted to adenine compounds by the nauplii, we exposed the larvae to ^{14}C-labeled purine mononucleotides and determined the specific activities of the acid-soluble purines and the nucleic acid purines. We observed that when ^{14}C-GMP is used it is converted to adenine nucleotides as well as guanine nucleotides, but, more significantly, the labeled guanine of the nucleotide pool serves as a precursor of nucleic acid guanine, whereas the adenine nucleotides derived from labeled guanine are not efficient precursors of nucleic acid adenine. This observation suggests that another unlabeled source of adenine is the major precursor of nucleic acid adenine. Use of ^{14}C-AMP and ^{14}C-IMP as precursors confirmed this conclusion. Thus the proposed unlabeled precursor of nucleic acid adenine must *not* mix with the acid-soluble pool of adenine to any great extent but is somehow compartmentalized. If diguanosine tetraphosphate serves as a source of adenine, as our previous results indicate, then it probably functions as the principal source of nucleic acid adenine and only secondarily as a source of acid-soluble adenine nucleotides.

To determine if the utilization of diguanosine tetraphosphate is linked to DNA synthesis in the nauplii, we exposed the larvae to ^{14}C-AMP and 5-fluorodeoxyuridine to inhibit DNA synthesis. We observed that the drug prevents a net increase in DNA adenine and strongly inhibits incorporation of ^{14}C-AMP into DNA while permitting incorporation of the isotope into RNA. An investigation of changes occurring in the nucleotide pool of control nauplii and nauplii treated with 5-fluorodeoxyuridine revealed that inhibition of DNA synthesis prevents utilization of diguanosine tetraphosphate and the net increase in ATP. However, the *turnover* of adenine in ATP is unaffected by the drug. This observation suggests once again that diguanosine tetraphosphate serves only as a secondary source of acid-soluble adenine, but now it appears that

its principal function is to supply adenine directly for DNA synthesis, not RNA synthesis.

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20.2 AN INVESTIGATION OF TREHALASE ACTIVITY IN *ARTEMIA SALINA*

R. D. Ewing

Dormant encysted embryos of the brine shrimp *Artemia salina* contain large amounts of the non-reducing disaccharide trehalose, which is used as an energy source for development of the embryo to the point of excystation. During this period, trehalose is rapidly degraded, with a concurrent synthesis of glycogen and glycerol. The rate of trehalose degradation has been found to be dependent upon the oxygen concentration of the medium, suggesting that the enzyme trehalase, which catalyzes trehalose hydrolysis, possesses regulatory properties. An investigation of trehalase activity in extracts of encysted embryos is now in progress. Optimal conditions for trehalose hydrolysis are presently being studied with the ultimate goal of examining possibilities of regulation by nucleotides.

21. Biochemical Regulation

F. T. Kenney[a]
W. D. Wicks
C. B. Hager[a,b]
J. R. Reel[a,b]
Claudio Pellegrino[c]
J. L. Wittliff[a,d]
D. D. Holten[e]
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Participation in Cooperative Programs:

AEC-NCI Cocarcinogenesis (26.40–26.45)

[a]Dual assignments
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21.1 HORMONAL REGULATION OF TYROSINE TRANSAMINASE IN FETAL RAT LIVER IN ORGAN CULTURE

W. D. Wicks

As discussed previously, although rat liver tyrosine-α-ketoglutarate transaminase is readily induced by hydrocortisone in adult rats, this enzyme is resistant to modification by the steroid in fetuses in utero.[1] Fetal rat liver has been placed in organ culture so that studies on the hormonal regulation of tyrosine transaminase can be carried out in an environment free of repressors of its synthesis presumed to operate during gestation.

As reported previously, hydrocortisone at 10^{-6} *M* had no effect on fresh explants of term fetal liver, but after 12 hr in culture a detectable increase in tyrosine transaminase activity was observed.[1] As the time in culture increased, this effect became progressively greater until by 48 hr the activity of the transaminase was four- to eightfold higher than that in control explants. If explants were cultured for 42 hr before hormone was added, only a brief lag was noted before enzyme activity increased, and the maximum induction (four- to eightfold) was achieved within 6 to 8 hr. These results are consistent with the conclusion that repressors of transaminase synthesis are present in fresh fetal liver which are presumably degraded during the early period in culture. Attempts to block steroid-mediated induction by extracts of fetal tissues have met with only partial success. Extracts of placenta do have a limited ability to prevent induction, but more experiments are required to assess the significance of this effect.

Although hydrocortisone has no detectable effect on either protein or RNA synthesis in the organ culture system, transaminase induction is abol-

ished by both cycloheximide and actinomycin D. Pulse labeling of tyrosine transaminase with high specific activity ^{14}C-leucine, as detected by selective immunochemical precipitation, showed that hydrocortisone increased the rate of transaminase synthesis threefold. These results demonstrate that an increase in the de novo synthesis of transaminase protein is responsible for the glucocorticoid-mediated elevation in enzyme activity in this system, as has been shown to be the case in adult rats in vivo.[2]

In the course of analyzing the specificity of hormonal induction of tyrosine transaminase in the organ culture system, it was found that some other hormones were effective inducers. Among various steroids tested, only the glucocorticoids were active, but the protein hormones glucagon and insulin each caused a limited induction of the enzyme, similar to observations in vivo.[3] Since these two hormones normally operate by opposing mechanisms, the possibility had to be considered that they induce the transaminase by different mechanisms. Because glucagon is a well-known stimulator of cyclic AMP formation in liver, it was of interest to test the ability of both cyclic AMP and epinephrine, an activator of hepatic adenyl cyclase, to induce the enzyme. Both substances proved to be as effective inducers as glucagon. Neither compound was effective when added directly to homogenates, indicating that activation of the transaminase was not involved. Cycloheximide completely abolished induction by these agents, implying that synthesis of new enzyme protein is responsible for their effect on transaminase activity. Dichloroisoproterenol, an inhibitor of the effects of epinephrine on cyclic AMP formation, completely abolished induction by the catecholamine but had no effect on that by hydrocortisone. These results support the conclusion that epinephrine induces tyrosine transaminase by way of cyclic AMP and suggest further that the latter substance induces by a mechanism distinct from that operative with hydrocortisone. Since glucagon also stimulates cyclic AMP formation in liver, it seems reasonable to conclude that it induces by way of the cyclic nucleotide as well. Since insulin is known to lower the hepatic level of cyclic AMP it would seem that insulin may induce the transaminase by yet another mechanism. Current experiments are aimed at directly investigating this possibility.

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21.2 HORMONE EFFECTS ON HYBRIDIZABLE RNA FROM RAT LIVER

Claudio Pellegrino F. T. Kenney

As detailed in other reports from this group, a variety of hormones change the rate of synthesis of the enzyme tyrosine transaminase in rat liver. At least two discrete induction mechanisms are now recognized, one of which is sensitive to hydrocortisone and the other to either of the polypeptide hormones insulin and glucagon. Drews and Brawerman have reported[1] that hydrocortisone treatment is associated with a qualitative change in the fraction of nuclear RNA that hybridizes with DNA; this fraction presumably contains the mRNA for transaminase synthesis. We are attempting to determine if the same change is detectable after treatment with insulin or glucagon. If such a change is found, it would indicate that there exist two mechanisms for promoting mRNA synthesis, but a negative result would suggest that the protein-hormone-mediated induction proceeds via a mechanism not involving stimulated mRNA synthesis. A great many problems were encountered in developing the hybridization technique to the point where competition experiments could be performed in a fashion that we consider to be meaningful. As this exploratory phase is now nearly complete, the hormone studies will be done soon as a joint venture with the Department of General Pathology of the University of Siena, Italy.

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21.3 HORMONAL CONTROL OF TYROSINE TRANSAMINASE IN ISOLATED, PERFUSED RAT LIVER

C. B. Hager G. R. Holloway F. T. Kenney

The rate of synthesis of hepatic tyrosine transaminase can be altered by the administration of any of several hormones to adrenalectomized rats. Hydrocortisone, insulin, or glucagon induces tyrosine transaminase, whereas treatment with growth hormone selectively represses this enzyme.[1] However, the in vivo approach is complicated by the fact that it is impossible to determine if an observed induction or repression occurs as a result of direct participation of the administered hormone at the tissue level. Consequently, the effect of each of the hormones known to alter the rate of tyrosine transaminase in vivo was studied in the isolated, perfused rat liver using the techniques of Miller *et al.*[2,3]

Hydrocortisone, insulin, and glucagon were found to increase tyrosine transaminase synthesis by direct action on the liver. A similar effect of hydrocortisone in the isolated, perfused rat liver has been previously reported.[4,5] However, repression by growth hormone is not observed in this system. Preliminary experiments indicate that growth hormone effects the release of some extrahepatic factor into the circulation, which in turn acts directly at the tissue level.

Induction of rat liver tyrosine transaminase apparently involves two separate mechanisms, both sensitive to actinomycin. The induction elicited by hydrocortisone continues for as long as the steroid is present in the perfusion medium. On the other hand, the elevated synthesis of tyrosine transaminase initiated by either pancreatic hormone ceases after 2 to 3 hr despite continuous hormonal infusion into the medium. These results support in vivo studies demonstrating additivity when hydrocortisone is administered with glucagon or insulin.[6]

Glucagon has been demonstrated to elevate the level of cyclic 3′,5′-AMP in liver preparations.[7] Therefore, the possibility of this nucleotide being involved as an intermediate in the induction of tyrosine transaminase is currently being investigated. Addition of the dibutyryl derivative of cyclic AMP elicits a slight increase in the activity of this enzyme that is kinetically similar to the response of either insulin or glucagon. However, the significance of this result is questionable since the maximum increase observed is not markedly different from that found in several control experiments. Epinephrine, which increases the level of cyclic AMP in the liver,[7] has not been used effectively because its vasoconstrictive effect prevents sufficient blood flow through the liver. However, both epinephrine and cyclic AMP are effective inducers of tyrosine transaminase in cultured explants of fetal rat liver.[8] Isoproterenol, an analog of epinephrine devoid of any vasoconstrictor activity, and theophylline, a potent inhibitor of the hepatic diesterase which inactivates cyclic AMP, are now being utilized in these studies.

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21.4 HORMONAL CONTROL OF RNA TRANSPORT

C. B. Hager F. T. Kenney

In vivo addition experiments[1] and studies carried out using isolated, perfused rat livers[2] have implicated at least two distinct mechanisms for the hormonal induction of rat liver tyrosine transaminase. The hydrocortisone-induced synthesis of this enzyme is maintained by the steroid[2–4] and presumably results from a shift in the spectrum of messenger RNA synthesis.[5] However, induction of tyrosine transaminase by either glucagon or insulin ceases after 2.5 to 3 hr, despite the presence of either protein hormone.[2]

Such a response may involve an alteration in the permeability of the nuclear membrane facilitating the transport of transaminase messenger. Assuming the existence of a messenger RNA pool in the nucleus, the induced synthesis of tyrosine transaminase would depend upon the lifetime of the stored messenger and not upon the presence of the inducers. Such a mechanism for induction by insulin and glucagon and for repression of tyrosine transaminase by growth hormone is currently being investigated.

The experimental procedure is designed to determine the effect of insulin, glucagon, and growth hormone upon the appearance in the cytoplasm of mRNA prelabeled in the nucleus with ^{3}H-orotic acid. The extent and time course of labeling of the 45 S particle, which is thought to be the transport form of mRNA,[6] and of polysomes are being studied. Preliminary experiments have been concerned with the resolution of these entities by density gradient centrifugation.

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21.5 STIMULATION OF LIVER RIBONUCLEIC ACID SYNTHESIS BY ESTROGEN

J. L. Wittliff F. T. Kenney R. A. Wallace[1]

For several years our laboratory has been interested in the effects of hormones on ribonucleic acid and protein synthesis. Induction of hepatic enzyme synthesis due to hydrocortisone is preceded by an increase in the rate of synthesis of three types of RNA:[2] (1) ribosomal precursor RNA, (2) DNA-like RNA, and (3) transfer RNA. This general effect on RNA synthesis after hormonal induction provides little opportunity for investigation of the mechanism of hormone action. However, Wallace and coworkers[3] have shown recently that administration of estrogen to male toads (*Xenopus laevis*) induces the formation of a serum lipophosphoprotein (SLPP), presumably synthesized in the liver. This lipoprotein complex may represent as much as one-half of the total serum protein when induction is maximal. Although estrogen may alter the levels of other liver constituents, the induction of a single species (SLPP) to such a concentration would appear to provide an ideal system for investigations of the mechanism of hormone action on the RNA level. Presently we are examining the labeling of the various types of RNA after estrogen treatment to determine the nature of the hormonal effect.

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22. Microbiology

Electron Transport Mechanisms in Microorganisms

S. F. Carson[a]
M. I. Dolin

[a]Dual assignments

22.1 KINETICS OF MALIC-LACTIC TRANSHYDROGENASE

M. I. Dolin

Malic-lactic transhydrogenase[1] catalyzes direct hydrogen transfer in the following reaction: malate + pyruvate $\rightleftharpoons$ lactate + oxalacetate. The steady-state kinetic behavior of the enzyme has been investigated in both directions, and rate equations describing the forward and reverse reactions have been derived. A consistent set of kinetic parameters has been obtained since (1) the theoretical rate equations for the forward and reverse reactions match the experimental data over a 100- to 200-fold range of substrate concentration and (2) the parameters for the forward and reverse reactions yield the equilibrium constant of the chemical reaction.

In a given direction, in the steady state, each substrate forms an abortive complex with the enzyme, and each product is an inhibitor of each substrate. This leads to 12 inhibition constants, 8 attributable to product inhibition and 4 to substrate inhibition. All inhibition constants have been evaluated, along with the other coefficients of the rate equations. It has been found that the dissociation constant for the abortive complex with pyruvate is the same as the dissociation constant for one of the two detectable spectral changes found on titration of the enzyme (E·DPNH) with pyruvate. In addition, the spectral change under consideration is one which is consistent with the formation of an unreactive enzyme–pyridine nucleotide–pyruvate complex.

In conjunction with other information, the steady-state kinetic data lead to the conclusion that the enzyme enters and leaves each catalytic cycle as a binary complex with a substrate molecule. Three general types of such a mechanism, all consistent with the empirical rate equation, can be written. For one particular type, it can be shown that the product terms of the rate equation yield the eight experimentally observed product inhibition constants. A more precise definition of the mechanism will require information concerning the distribution of enzyme species in the steady state.

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23. Plant Physiology and Photosynthesis

Plant Physiology (23.1–23.6)		Photosynthesis (23.7)	
A. H. Haber	Sarah A. Angel	W. A. Arnold	J. R. Azzi
D. E. Foard	Pat C. Kerr	W. F. Bertsch	C. W. Smith
Patricia L. Walne[a]	Nina S. Hammer[c]	R. M. Pearlstein	
D. H. O'Dell[b]	Helen J. Luippold[a]		
	Rhonda G. Farmer		
	Paula J. Thompson[d]		
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23.1 LIGHT RETARDATION OF FUNCTIONAL AND ULTRASTRUCTURAL CHLOROPLAST DETERIORATION AND ITS INDEPENDENCE FROM PHOTOSYNTHESIS

A. H. Haber
Paula J. Thompson
Patricia L. Walne
L. L. Triplett

It is common knowledge that leaves are greener in the light than in the dark and that in most higher plants light is necessary for the formation of the photosynthetic apparatus. The work described in this report was begun to see to what extent, if any, light could control the photosynthetic capacity of leaves by also controlling functional and ultrastructural aspects of senescence of chloroplasts already formed. Such a study of chloroplast and chlorophyll degradation is made feasible by use of aminotriazole, a chemical which prevents chlorophyll synthesis and normal chloroplast formation even in the light.[1]

Aminotriazole treatments were found which permitted wheat leaf growth without permitting any detectable chlorophyll synthesis, as shown by the complete absence of any chlorophyll peak in acetone extracts of those portions of the leaf formed subsequently. In one experiment, described below, we also used electron microscopy to confirm the absence of chloroplast formation. Subsequent experiments were performed with excised apical portions of green leaves incubated with appropriate concentrations of aminotriazole either in darkness or under white light (Gro-Lux bulbs) of various intensities and photoperiods. The leaf sections were analyzed for chlorophyll content for periods of from two to eight days after excision. During this time the decline in chlorophyll content, identical with chlorophyll decay since chlorophyll synthesis was absent, was less in light than in darkness. Better chlorophyll retention in the light was accompanied by a greater capacity for light-dependent ^{14}C fixation from labeled bicarbonate relative to controls that had been in darkness. When photosynthetic fixation could occur in the light, there was an optimal light exposure for the effect on chlorophyll retention. When photosynthesis was prevented by the photosynthetic poison DCMU, however, the effect showed light saturation, and continuous high light intensities were more effective than in the absence of DCMU. These findings suggest that products of photosynthesis accelerate chloro-

plast deterioration and can counteract a nonphotosynthetic effect of light itself at high intensities. This suggestion is corroborated by experiments involving application of carbohydrate either in light or in darkness. An electron microscope study showed that under saturating light intensities and with photosynthesis prevented by DCMU, the ultrastructural disintegration of the chloroplasts was much slower in light than in darkness. There was no ultrastructural evidence for chloroplast formation in these experiments, as expected, since aminotriazole was also present. Under continuous illumination, light saturation for the effect was at about 2400 ergs cm^{-2} sec^{-1}, and some effect was detected with only 9 ergs cm^{-2} sec^{-1}.

A light requirement for chloroplast formation is well known. Our results indicate that light has a dual control in maintaining the green status of the plant by exerting also a second effect: retardation of senescence of chloroplasts already present. Just as the light effect on chlorophyll formation does not require photosynthesis,[2] our results show that the light effect on retarding senescence of chloroplasts already present also does not require functional photosynthesis.

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23.2 CANCELING ERRORS IN THE QUANTITATIVE DETERMINATION OF α-AMYLASE ACTIVITY

A. H. Haber L. L. Triplett

Stimulated interest in amylases followed recent demonstrations that de novo synthesis of α-amylase is triggered by gibberellin.[1] A method often used for determining the activity (effective concentration) of α-amylase depends upon its capacity to hydrolyze α-1,4-glucosidic bonds with a consequent decreased capacity of starch to form a blue color with iodine. Applications of this method have been based on α-amylase activity being proportional to the decrease in starch-iodine absorbancy and inversely proportional to reaction time.[1-6] This report indicates, however, that under conditions in which starch-iodine absorbancy is initially proportional to starch concentration in the absence of any enzyme action, subsequent decreases in absorbancy resulting from α-amylase action are not proportional to enzyme concentration and reaction time. We also discuss (1) a possible explanation for the previous findings that decreases in starch-iodine absorbancy seem proportional to α-amylase concentration and reaction time and (2) suggestions for quantitatively determining α-amylase activity by starch-iodine absorbancy changes despite these difficulties.

We find that for any given level of iodine reagent, absorbancy at 620 nm is proportional to low starch concentrations only. Proportionality at higher starch concentrations appears to be prevented by depletion of the iodine reagent. Studies described in the next paragraph were performed using starch and iodine reagent so that absorbancy decreased linearly with starch dilution.

When a given concentration of highly purified and crystallized α-amylase was incubated with the soluble starch, the absorbancy decreased nonlinearly with increasing time, enzyme concentration, and product of reaction time and enzyme concentration. The observed decrease in absorbancy is proportionately greater for smaller than for larger values of the product of enzyme concentration and reaction time. Consequently, there is no true constant of proportionality; that is, the change in absorbancy is not proportional to enzyme concentration and reaction time. Despite this conclusion, for any given absorbancy change enzyme concentration and reaction time are still inversely proportional to each other. The nonconstancy of absorbancy change per unit of the product of enzyme concentration and reaction time could readily be explained by assuming that the color yield of starch-iodine complex per unit length of starch molecule decreases with decreasing chain length, as has been suggested.[7]

The question remains, why have others found that absorbancy changes seem proportional to enzyme concentration and reaction time? One explanation might be that initial starch concentrations were so great that, apart from any enzyme action, dilution produced the less than proportionate decreases in absorbancy described in the first paragraph of this section. Thus the absolute

value of the absorbancy change per unit decrease in starch concentration would be a monotonically *increasing* function of the decrease in starch concentration. In the absence of this error, however, as already stated, the presumed constant of proportionality relating absorbancy change to the product of enzyme concentration and reaction time is a monotonically *decreasing* function of the product of reaction time and enzyme concentration. Consequently, the two disporportionate aspects of absorbancy change tend to cancel each other out.

These results indicate that changes in starch-iodine absorbancy are not quantitative measures of the progress of α-amylase hydrolysis of glucosidic bonds. Since truly quantitative determinations should not depend upon canceling errors, quantitative assay should not be based upon the magnitude of the absorbancy change but upon the constant product of enzyme concentration and reaction time to produce a given absorbancy change.

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23.3 IMMEDIATE AND DELAYED EFFECTS OF LETHAL DOSES OF GAMMA RADIATION ON ASPECTS OF PHOTOSYNTHESIS AND ON ULTRASTRUCTURE OF CHLOROPLASTS AND OTHER ORGANELLES

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A. H. Haber J. R. Azzi[1]

We have shown that a 1-megarad acute lethal dose of gamma irradiation given to green wheat leaf tissue produces no immediate effect on chloroplast ultrastructure and actually suppresses greatly the normal disintegration of chloroplasts in darkness.[2,3] The work described in this report was undertaken, using methods described earlier,[2] to answer the following questions: (1) Except for the slower time scale, does the sequence of events observed in ultrastructural disintegration of chloroplasts in darkness after 1 megarad resemble the sequence observed in non-gamma-irradiated controls? (2) Is there an optimal dose for preservation of chloroplast ultrastructure? (3) To what extent are the radiation effects on chloroplast ultrastructure accompanied by radiation effects on aspects of function in and close to the photosynthetic pigment system? (4) Are the immediate and delayed effects on chloroplasts paralleled by similar effects on ultrastructure of other cellular organelles? Also discussed is the possible relevance of these findings to pigment localization by electron microscope studies.

The answer to the first question in the preceding paragraph appears to be yes. (A description of the events in ultrastructural disintegration of chloroplasts in non-gamma-irradiated controls in darkness is given in the earlier report.[2])

In our experiments 1 megarad appears to be at or near an optimal dose for preservation of chloroplast ultrastructure. Doses too far below 1 megarad are sublethal and permit normal chloroplast disintegration; doses too far above 1 megarad, for example, 2 megarads, produce immediate chloroplast damage, even when the irradiation is performed at 0°C in darkness.

The 1-megarad dose, which retards ultrastructural disintegration of chloroplasts in darkness during four days after irradiation, also preserves their capacity to emit "glow curves"[4] after illumination with white light at −196°C. After 2 megarads the glow curves appear normal when assayed immediately after irradiation but are completely abolished after four days in darkness. Their capacity to emit delayed light normally was prevented immediately after 1 megarad. A flattened decay curve with abolished fast decay components was found. Such a curve is also found in *Chlorella* treated with the photosynthetic poisons DCMU, CMU, and orthophenanthroline[5] and in our green wheat leaf tissue treated with DCMU. The glow-curve results suggest that the "optimal dose" (1 megarad) that preserves chloroplast ultrastructure also preserves the capacity to put energy from absorbed light into electron traps in the photosynthetic pigment system exclusive of

subsequent enzyme reactions. This capacity is lost within a few days after the higher radiation dose (2 megarads) that itself produces very early structural damage observable by electron microscopy. The effects of these different lethal doses on chloroplast ultrastructure and on the functioning of the photosynthetic pigment system exclusive of enzyme reactions are thus correlated. By contrast, the delayed-light results indicate that even the dose that preserves ultrastructure very quickly prevents normal energy storage in the photosynthetic system coupled to the enzymatic reactions of electron transport.[6]

The absence of immediate detectable effects from 1 megarad, the reduction in rate of disintegration after 1 megarad relative to that in non-gamma-irradiated controls, and the presence of immediate structural damage after 2 megarads, which three ultrastructural effects we find in chloroplasts, we find also in the plasma membrane, tonoplast, dictyosomes, endoplasmic reticulum, mitochondria, nucleus, and ribosomes.

The 1-megarad-induced prevention of normal chloroplast disintegration in darkness contrasts with the 1-megarad-induced acceleration of destruction of chlorophyll that occurs with illumination.[3] Physiological and ultrastructural work is in progress to study this photodestruction itself, so as to permit a somewhat direct indication of pigment localization within the chloroplast. Preliminary results indicate that the ultrastructural sequence of events that accompanies photodestruction of chloroplasts after the 1-megarad dose appears to be qualitatively different from the sequence of events that occurs during normal chloroplast disintegration in darkness or after 1 megarad in darkness. Consequently, a characterization of the locus of light action uncomplicated by either normal or gamma-radiation-induced chloroplast disintegration may be possible.

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23.4 INDUCED MITOTIC ACTIVITY AND DISORGANIZED GROWTH IN RELATION TO AUXIN-INDUCED TUMORS

D. E. Foard Helen J. Luippold
A. H. Haber Rhonda G. Farmer

Continuing our studies on the relation between cell divisions and disorganized growth, we investigated the effects of high concentrations of auxin on parenchyma, the main tissue in which plant tumors arise. Previously we have studied the effects of high concentrations of auxin on parenchyma from nonshoot structures of the wheat embryo. This report concerns the induction by auxins of (1) mitoses in parenchyma of the epiblast-coleorhiza, a structure that normally grows without mitoses, and (2) excessive, disorganized growth of the cortical parenchyma of the coleoptile, a shoot structure.

When unirradiated wheat grains are sown on high concentrations (2×10^{-3}, 10^{-3}, or 10^{-4} M) of the auxin indoleacetic acid (IAA), the epiblast-coleorhiza, which is all parenchyma except for a single layer of epidermis, grows in an excessive, disorganized fashion. On the first day after sowing on the high auxin concentrations, a small number of mitoses occur in the parenchyma of the epiblast-coleorhiza. Mitoses increase in frequency on the second day and reach a peak on the third day after sowing. Mitotic activity is negligible or absent by the fourth and fifth day after sowing. Sections of the epiblast-coleorhiza reveal that the mitotic activity is localized primarily in a zone near the periphery. Kinetin in the presence of auxin may enhance the mitotic activity, although kinetin without auxin does not induce such activity by itself. This is the first demonstration ever of mitosis occurring in the epiblast-coleorhiza of any grass during germination. The inducibility of mitotic activity in the epiblast-coleorihiza, in addition to the previously demonstrated inducibility of disorganized growth after normal growth has ceased,[1] further strengthens the analogy to tumor induction in plants.

Treatment with the auxin naphthaleneacetic acid (10^{-3} M) causes tumor-like growths in the basal portion of the coleoptile of "gamma plantlets" (seedlings which grow without divisions after exposure of the grain to gamma irradiation). Sections of such coleoptiles reveal that the excessive, disorganized growth occurs mainly in the cortical parenchyma. The epidermis is affected to a small degree; the vascular tissue shows no histological disturbances. The tumor-like region has histological features similar to those previously described for the epiblast-coleorhiza and root cortical parenchyma of IAA-treated gamma plantlets.[2] Thus the induction of tumor-like growth has been demonstrated for a shoot part of wheat seedlings growing without divisions and now includes structures which normally grow with many (root), few (coleoptile), and no (epiblast-coleorhiza) cell divisions.

The finding of auxin-induced tumor-like growths in the coleoptiles of gamma plantlets, but not of seedlings from unirradiated grain, is similar to our previous finding with roots.[2] Perhaps the inducibility in gamma plantlets might be explained by an accelerated ontogeny resulting from mitotic inhibition in those organs that normally would have grown with cell divisions during germination. By contrast, the epiblast-coleorhiza, inducible even in seedlings from unirradiated grain, is near the end of its ontogeny irrespective of irradiation. Auxin inducibility of tumor-like growths in three different structures of both gamma plantlets and unirradiated seedlings may be summarized in the following generalizations: (1) The inducibility is not positively correlated with either mitotic activity or irradiation and (2) the inducibility is positively correlated with the proximity of an organ to the end of its development.

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23.5 RADIATION-INDUCED CELL LETHALITY AND SHOOT MERISTEM COLLAPSE IN THE ABSENCE OF MITOSIS

D. E. Foard — Sarah A. Angel
A. H. Haber — Pat C. Kerr

The absence of cell lethality and meristem collapse in maize roots after high doses of ionizing radiation to the seed has been attributed to the masking of chromosomal damage by the radiation-induced inhibition of cell division.[1] Thus far our findings on gamma plantlets (wheat seedlings growing without cell divisions after heavy gamma irradiation of the grain) are consistent with this idea.[2,3,4] In order to test this idea further, we have irradiated both growing gamma plantlets and seedlings growing from unirradiated grain and have studied them histologically. A previous report considered the root;[4] the present report considers the shoot.

Seedlings from unirradiated grain and gamma plantlets from grain given 800 kilorads were irradiated when the coleoptile was about 3 mm long with gamma-ray doses more than sufficient to produce chromosome breakage. After shoot growth had ceased, these plants were fixed and histological sections were prepared. We also fixed and prepared gamma plantlets and seedlings from unirradiated grain, both sets of which received no seedling irradiation. The results are as follows: (1) 0 kilorad to dry seed; 0, 25, 50, or 100 kilorads to seedling: In the plants that received neither seed nor seedling irradiation, the stem apex and leaf primordia have typically immature features and many mitoses; new leaf primordia are initiated; nuclear and cell divisions occur in the subapical region of the stem. In the irradiated seedlings the stem apex and leaf primordia are neither necrotic nor collapsed, but have mature features, especially vacuolate cells, and no evidence of cell divisions; no new leaf primordia appear; the subapical region of the stem consists of very elongate cells. These plants look surprisingly similar to gamma plantlets. (2) 800 kilorads to dry seed; 0, 5, 10, 15, or 20 kilorads to seedling:

The stem apex and leaf primordia are neither necrotic nor collapsed, but have mature features, especially vacuolate cells, and no evidence of cell divisions. The subapical region of the stem consists of elongate cells. Stems of gamma plantlets reirradiated as seedlings do not become as long as those of gamma plantlets not reirradiated as seedlings. These results were found in the same experiments in which reirradiation stimulated gamma-plantlet leaf growth.[5] (3) 800 kilorads to dry seed; 100 or 200 kilorads to seedling: The stem apex shows signs of incipient necrosis and collapse; youngest leaf primordium in all plants is necrotic, and outer cell layers are collapsed; cells in the subapical portion of the stem are vacuolate but are neither collapsed nor elongate. (4) 800 kilorads to dry seed; 400, 500, or 600 kilorads to seedling: The stem apex is necrotic, and its peripheral cell layers are collapsed; youngest leaf primordium is collapsed; the next youngest leaf primordium is necrotic and partially collapsed; cells in the subapical portion of the stem are vacuolate, but are neither collapsed nor elongate.

Results in 1 above suggest that the histological features observed in irradiated seedlings growing from unirradiated grain are a consequence of the inhibition of cell division. The results in 2 above indicate that relatively low irradiations of gamma plantlets affect stem growth without causing cell lethality and meristem collapse. The results in 3 and 4 above indicate that, if the dose be high enough, radiation-induced cell lethality and meristem collapse can occur in the absence of genic imbalance due to chromosomal aberrations in dividing cells. A point of particular interest here is that 100 kilorads seedling irradiation appears to produce more shoot damage in a gamma plantlet than in a seedling from unirradiated grain. This observation is somewhat unexpected, based on our previous observations of roots.[4]

The results in this and related previous reports[2,3,4] indicate that the root is more radiosensitive than the shoot after both seed and seedling irradiation. After 100 kilorads of gamma irradiation to seedlings from unirradiated grain, the shoots subsequently look as if they were gamma plantlets in two respects: the absence of cell lethality and the presence of anatomic features similar to gamma plantlets. Roots of such seedlings, on the other hand, show cell lethality and collapsed meristems, presumably from genic imbalance due to chromosome breakage in dividing cells. For given doses of seedling irradiation, the gamma-plantlet root appears more radioresistant, whereas the gamma-plantlet shoot appears more radiosensitive than the corresponding structures in seedlings from unirradiated grain. This apparent paradox might be reconciled by the observation that after irradiation of seedlings growing from unirradiated grain, only shoots appear to behave as they do in gamma plantlets; that is, there is an apparent masking of chromosomal damage by the radiation-induced inhibition of cell division.

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23.6 USING COLCHICINE TO DETERMINE THE NUMBER OF CELLS INITIALLY FORMING A LATERAL ROOT PRIMORDIUM

D. H. O'Dell D. E. Foard
Rhonda G. Farmer

The question of how many cells are involved in the initiation of an organ primordium is often difficult to answer because the region of origin may be diffuse and because accompanying cell divisions may obscure the originally participating cells. The first difficulty may be overcome by studying a primordium, for example, a lateral root primordium, that regularly arises in a localized region of a single cell layer. The second difficulty may be overcome by using an agent, for example, colchicine, that prevents cell division without simultaneously preventing initiation of primordia.

In the lateral root primordia of seedlings from embryos germinated on colchicine, pericyclic

cells that normally would have divided instead replicate their chromosomes without nuclear division. Cells in the lateral root primordium thus become "tagged" by their polyploid restitution nuclei. Therefore it is possible to count directly from serial sections the number of parent-root pericyclic cells involved in the initial formation of a lateral root primordium. Thus far such counts have been made for three cereals: wheat, rye, and barley. In each case approximately 16 pericyclic cells are involved in the initial formation of a lateral root primordium. This investigation is currently being extended to species in other groups.

23.7 PHOTOSYNTHESIS

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W. F. Bertsch[1] J. R. Azzi
R. M. Pearlstein

We are studying the mechanism that the green plant uses to convert the energy of light quanta into chemical oxidizing and reducing power. Delayed light has been our main tool. During 1966 two experiments were made that justify this use of delayed light. Dr. L. Jones, of the University of Tennessee, spent the summer in Oak Ridge. He showed that in completely relaxed plants two absorption acts are required for the emission of delayed light. At the same time Dr. W. Bertsch was visiting the laboratory of Dr. R. Hill in Cambridge. He, together with Dr. R. Hill and Dr. Janet West, showed that in chloroplasts the activation of noncyclic electron transport resulted in an increased emission of delayed light at 1 msec.

Before these two experiments, few people had doubts that delayed light production and photosynthesis were competitive. It is clear that the energy of one quantum cannot be used for both delayed light emission and photosynthesis. It is now clear that the energy of two quanta can, by some kind of cooperation, do both.

Delayed Light Studies on Chloroplasts. – In the early part of this year the Cambridge experiment was repeated a number of times in Oak Ridge. The results are being published. In addition, we also studied changes in the fluorescence intensity of chloroplasts as noncyclic electron transport was activated.

Energy Storage at Low Temperature. – Frozen green plants are able to store energy, as is shown by the reemission of light on heating the cells to 100°C. Five different kinds of light emission can be seen. As the sample is heated from –196 to 100°C, four glow curves (a glow curve is a spike of light emission at a certain temperature) and a general low emission in the temperature range of 80 to 100°C are found. One of the glow curves has the properties that are to be expected for the first energy-storing step in photosynthesis:

1. The absorption cross section is more than 100 times that of a chlorophyll molecule, thus agreeing with the size of the photosynthetic unit.
2. Previous heating to 55°C for 5 min removes the peak. This treatment is known to inactivate system II.
3. The mutants of Dr. Bishop show that this glow curve is coming from system II.
4. DCMU, 3-(4-chlorophenyl)-1,1-dimethyl urea, treatment makes this glow curve larger. DCMU is believed to stop electron transfer.
5. With chloroplasts, ferricyanide is known to be an electron acceptor for the Hill reaction. Ferricyanide makes this glow curve smaller.
6. The activation energy for this glow curve appears to be about 0.6 ev.

Decay Profile of Beta-Particle-Excited Fluorescence of Chlorophyll in Green Plants. – Work begun last year to determine the decay profile of chlorophyll fluorescence in vivo is continuing. We have determined that green leaves luminesce under excitation by beta particles. The luminescence yield is at least 0.4 photon per incident beta particle from a ^{147}Pm source. The integral luminescence spectrum excited by the beta particles closely resembles the integral chlorophyll fluorescence spectrum excited by blue light. The luminescence yield of 0.4, together with reasonable assumptions, implies that the passage of one beta particle through a leaf excites some 160 molecules of chlorophyll a. With these encouraging results, apparatus was constructed in the Biology Division for use with a ^{90}Sr source for fast-timing studies. The apparatus and ^{90}Sr source were assembled at the Neutron Physics Division, with whom we collaborated on the initial development of the fast-timing electronics. Using Cerenkov radiation from a thin piece of plastic (instead of a leaf) as a test, we obtained a coincidence resolving time of 0.7 nsec from the un-

refined instrument. The instrument now will be set up in the Biology Division. With some further development work we expect to achieve a resolution close to 0.1 nsec.

Exciton Trapping in Photosynthetic Units and Related Systems. – During the past year new theoretical studies of the exciton trapping problem were initiated. In earlier studies[3] only weak energy transfer coupling between molecules was considered. In the present studies we consider strong coupling. The concept of a standing-wave exciton is introduced.[4] Exact solutions have so far been obtained for the rate of trapping a standing-wave exciton from a simple linear polymer with a trap at one end. Surprisingly, we find that strong coupling can lead to a slower rate of trapping in such a polymer than weak coupling. On the other hand, strong coupling may lead to faster trapping than weak coupling in photosynthetic units. We intend to continue these studies, and we hope to coordinate them with our experiments on chlorophyll fluorescence and delayed light.

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24. Microbial Photosynthesis

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Opal K. York

[a]Dual assignments
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24.1 THE MOLECULAR DIVERSITY OF THE PRIMARY PHOTOSYNTHETIC CARBOXYLATION ENZYME

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R. C. Fuller

In higher green plants "fraction I," a high-molecular-weight compound with $S_{20,w}$ of 18, ribulose 1,5-diP carboxylase, and "holochrome," a chlorophyll-A–protein complex, have been shown to be closely related or identical.

The purpose of the present experiments was to ascertain whether or not "fraction I" or some similar protein component with carboxylase activity could be observed in extracts of the green alga *Euglena gracilis*, an organism which contains chloroplasts, and in extracts of the procaryotic blue-green alga *Anacystis nidulans*. In addition, a comparison was made of the properties of the carboxylases of the photosynthetic bacteria *Rhodospirillum rubrum, Rhodopseudomonas spheroides, Rhodopseudomonas palustris*, and *Chromatium*, organisms which contain ribulose diphosphate carboxylase, but which do not have the eucaryotic cell structure.

An 18S peak was observed in schlieren diagrams of extracts of light-, but not in dark-grown *Euglena*. The S values obtained for the ribulose 1,5-diP carboxylases from *Euglena, Anacystis*, and *Chromatium* ranged from 18 to 20 (see Table 24.1.1). In sharp contrast, two distinct and different ribulose 1,5-diP carboxylases are found in a third and possibly more primitive group of photosynthetic bacteria, the *Athiorhodaciae*. The carboxylases from *R. palustris* and *R. spheroides* have S values of 12 and 14.5 (Table 24.1.1). It is interesting that the dimers of 12 and 14.5S proteins would have S values of 17 and 20. One explanation of these data could be that the 18 to 21S enzyme found in higher plants, algae, and *Chromatium* is a stable dimer of the carboxylase found in *Rhodopseudomonas*. It is apparent, in

Table 24.1.1. S_{rel} Values for Ribulose 1,5-diP Carboxylases from Various Photosynthetic Organisms

Organism	S_{rel} Values[a]
Rhodospirillum rubrum	6.2
Rhodopseudomonas palustris	12
Rhodopseudomonas spheroides	14.5
Chromatium, strain D	18
Anacystis nidulans	19.5
Euglena gracilis	19

[a]Values, obtained as described in the text, are the average of at least two determinations with extracts from different cultures of cells.

any case, that "fraction I" (or 18 to 20S ribulose 1,5-diP carboxylase) is not characteristic of all photosynthetic organisms.

Neither an 18 to 20S fraction nor a 12 to 14.5S fraction was found in extracts of *Rhodospirillum rubrum*. The S_{rel} value, in contrast, of the ribulose 1,5-diP carboxylase from *R. rubrum* was 6.2 (Table 24.1.1). Clearly, this carboxylase is physically distinct both from chloroplast "fraction I" and from the carboxylase of *R. spheroides* and *R. palustris*.

Although the complete details of the mechanism of the primary photosynthetic CO_2 fixation reaction from ribulose 1,5-diP and CO_2 to two moles of 3-P-glyceric acid, catalyzed by ribulose 1,5-diP carboxylase, are not currently known, one can assume that the overall reaction is similar, if not identical, in the multiplicity of organisms which carry out photosynthesis. We are, therefore, faced with a situation where the same or a very similar reaction is catalyzed by an enzyme in at least three quite different molecular forms. Additional experiments are required to determine whether or not the three carboxylases (6S, 13S, and 18S) are indeed similar proteins or chemically unrelated.

Reference

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24.2 PTERIDINES IN PHOTOSYNTHESIS

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The primary electron acceptor in photosynthesis has not been clearly delineated. Ferrodoxin, up until recently, was the compound with the lowest biological potential (−0.4 mv) to be implicated in photosynthetic electron transport. However, the primary photochemical act in system I of plants and the single photochemical act in bacterial systems can generate a low-potential reductant of around −0.8 mv.

The tendency of 6-substituted unconjugated pteridines similar to biopterin to increase in amount parallel to chlorophyll in developing photosynthetic systems, indicates a role for pteridines in photosynthesis. This correlation has been observed in photosynthetic bacteria, algae, and higher plants. The association of pteridines with the photochemical apparatus and the low redox potentials (−0.700 to −1.200 v) of these compounds suggest a role in electron transport that may be related to the production of a low-potential reductant in system I in green plants or to the single photochemical act in bacteria.

A specific inhibitor of pteridine utilization in *Tetrahymena* and *Crithidia*, 4-phenoxy-2,6-diaminopyridine (PDAP) (Fig. 24.2.1), inhibited photophosphorylation by chromatophore preparations and CO_2 fixation by whole cells. The development of the photosynthetic system of *Rhodospirillum rubrum* was also inhibited by PDAP. This effect was reversible by addition of a purified pteridine fraction and also by addition of biopterin.

A secondary effect of PDAP was to inhibit colored carotenoid synthesis. This effect was not reversed by addition of pteridine. The effect of PDAP on carotenoid synthesis was similar to the action of diphenylamine in that the more saturated phytoenes accumulate in place of the normal carotenoids. The data suggest an inhibition of the dehydrogenation of phytoene by pteridine inhibitors.

Chemical analysis of the pteridines isolated from these bacteria indicates that they are derivatives of 2-amino-4-hydroxy-6-(2′,3′-dihydroxypropyl)pteridine biopterin. The role of these compounds in photosynthetic electron transport is being further investigated.

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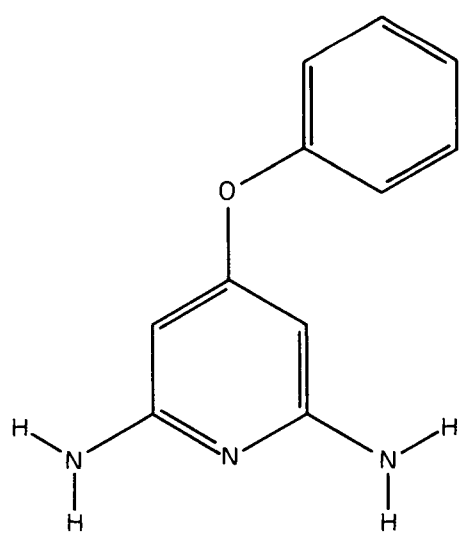


Fig. 24.2.1. 4-Phenoxy-2,6-diaminopyridine (PDAP).

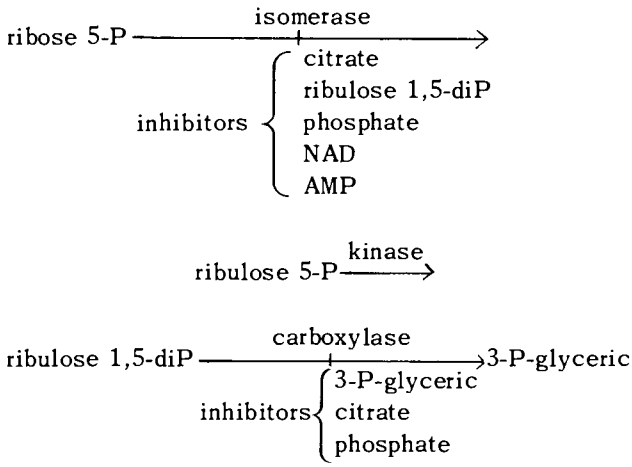
24.3 REGULATION OF ACTIVITY OF CALVIN CYCLE ENZYMES IN *RHODOSPIRILLUM RUBRUM*

Louise E. Anderson R. C. Fuller

Three reductive pentose phosphate pathway enzymes, ribose 5-P isomerase, ribulose 5-P kinase, and ribulose 1,5-diP carboxylase, catalyze the formation of phosphoglyceric acid from ribose 5-P and CO_2 in green plants and in photosynthetic bacteria. In *Rhodospirillum rubrum*, high levels of kinase and carboxylase activity are induced by autotrophic growth conditions, whereas isomerase levels remain relatively constant regardless of the mode of growth.

The isomerase and carboxylase have been purified from *R. rubrum*. The isomerase has a molecular weight of 57,000. The K_m for ribose 5-P is 1.6×10^{-3} *M*. The isomerase reaction, measured in the direction ribose 5-P to ribulose 5-P, is inhibited by citrate, ribulose 1,5-diP (but not by ribose 5-P), phosphate, NAD, and AMP. The carboxylase has a molecular weight of 120,000. The K_m for ribulose 1,5-diP is 1.2×10^{-4} *M*; for CO_2, 2.3×10^{-2} *M*. The carboxylase is inhibited by a 3-P-glyceric acid, citrate, and phosphate. It is not inhibited by excess ribulose 1,5-diP or by ribose 5-P.

The following reactions show the sites of inhibitions of various compounds:



These data indicate that ribose 5-P isomerase plays a key role in the regulation of photosynthetic CO_2 metabolism in this organism. The control of ribose 5-P isomerase activity, but not of ribulose 1,5-diP carboxylase activity, appears to be intimately linked to compounds involved in energy metabolism (AMP and NAD). It is suggested that light, through the formation of ATP and NADH, is involved both directly and indirectly in the operation and regulation of the reductive pentose phosphate pathway.

25. Biophysics

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Excited-State Biophysics

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R. O. Rahn
J. A. Knopp[b]
J. J. ten Bosch[b,c]
J. L. Hosszu
R. I. Fox[d]
Linda L. Peterson[d]
Maria Del Carmen Battista

Molecular Biophysics

L. G. Caro
Ann B. Jacobson
R. A. Weisberg
Dorothy M. Skinner
Grete Kellenberger Gujer[c]
R. W. Williams
D. P. Allison
Peggy P. Hester
L. L. Triplett
Anne-Marine Tucker[d]
Phyllis Newport Atkins

Radiation Biophysics

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J. S. Cook[a,e]
J. E. Donnellan, Jr.
R. A. McGrath[f]
P. A. Swenson
Thomas Sproston[g]
M. P. Gordon[h]
Susuma Takeda
W. L. Carrier
Janet R. McGrath
R. S. Stafford
Barbara A. Hamkalo[i]
A. J. Zelnis

X-Ray Diffraction

J. R. Einstein[a]
F. C. Hartman[a]
C. H. Wei[a]
I. Lucile Norton[a]

Participation in Cooperative Programs:

AEC-NCI Cocarcinogenesis (26.17-26.26)

[a]Dual assignments
[b]Biology Division postdoctoral investigator
[c]Visiting investigator from abroad
[d]Student trainee
[e]Consultant
[f]Temporarily at Donner Laboratory, University of California, Berkeley
[g]On sabbatical leave from the University of Vermont, Burlington
[h]On sabbatical leave from the University of Washington, Seattle
[i]ORAU Predoctoral Fellow

25.1 CHROMOSOME REPLICATION IN *ESCHERICHIA COLI*

L. G. Caro Claire M. Berg[1]

It has been stated by Nagata and other investigators that the integration of the sex factor in the chromosome determines the direction and origin of its replication in Hfr strains of *E. coli*.

The relative gene frequency in isogenic Hfr strains of *Escherichia coli* K-12 was assayed by transduction with the phage P1 in order to evaluate the influence of the integrated F factor on the origin and direction of chromosome replication. It is expected that genes located near the origin of replication will give approximately twice as many transducing particles as those located near the end. Three Hfr strains, with different sites of

integration of the F factor and different directions of chromosome transfer in conjugation, were studied. No significant differences in transduced gene frequency were found in one-cycle lysates made on exponential cultures of the strains. We labeled the chromosomes of the same strains with a pulse of 5-bromouracil and prepared P1 lysates on the cells thus labeled. Heavy transducing particles, having picked up the labeled portion of the chromosome, were separated by density gradient centrifugation in CsCl and analyzed for the frequency of transduction of various markers. In an exponential culture it is expected that the position of the replication point will show a preference for the origin of replication. However, no difference in transducing frequency for various markers is found between the three strains.

We concluded that, in these strains, the integrated F factor does not affect the origin or the direction of chromosome replication.

In an effort to determine whether the origin of chromosome replication is a stable genetic site, cultures of *E. coli* K-12 thy$^-$ leu$^-$ were starved for leucine and labeled with 5-bromouracil during the last 20 min of residual DNA synthesis, during the first 20 min after the amino acid was restored, and during 15 min of exponential growth. Gene frequency was analyzed in the hybrid-density transducing particles found in a P1 lysate made on these cells just after labeling. The results show a definite gradient of marker frequency running in opposite directions for the first two types of labeling, as compared with the control made on an exponential culture. They show that, for several Hfr strains derived from *E. coli* K-12 W1485, replication stops in a region located between ade_3 and *try* and runs in a counterclockwise direction. Results from the analysis of total lysates on cultures lined up by amino acid starvation indicate that the stopping point may correspond to the origin of replication.

A different line of strains, derived from *E. coli* K-12 CR-34, shows a different origin, located between *lys* and *metE* and a *clockwise* direction of replication.

We have, therefore, shown that, as had been postulated, amino acid starvation stops chromosome replication at a fixed site. It is likely that this site is the origin of replication. It is stable but may be strain specific. Further efforts will be directed toward studying the properties of this site and the control of chromosome and episome replication.

Reference

[1]Institut de Biologie Moléculaire, Université de Genève, Switzerland.

25.2 THE INCORPORATION OF 5-BROMOURACIL IN THE CHROMOSOME OF *ESCHERICHIA COLI* K12

L. G. Caro Claire M. Berg[1]
D. P. Allison

Much of our knowledge of DNA replication in *Escherichia coli* comes from the strain 15T$^-$. In the course of our experiments we found some differences between K12 strains and 15T$^-$. The major difference, from our point of view, is the existence in K12 of a fairly large thymine pool, not exchangeable with exogenous thymine, the complete exhaustion of which, by starvation, takes almost one full generation time. This pool makes short pulses of label very difficult to interpret, a fact which must be kept in mind in the design of experiments.

Many experiments on chromosome replication in *E. coli* involve labeling with bromouracil. Accordingly we have tried to see if BU affects the pattern of DNA replication in the strains we use. Unfortunately, experiments similar to those used by Lark to demonstrate sequential replication of the chromosome in 15T$^-$ are made impossible by the existence of the thymine pool mentioned earlier. We had to resort to other types of experiments.

In one type we labeled two aliquots of an exponential culture with either tritiated BU or tritiated thymine. After 15 min we washed the culture by filtration and placed it in cold thymine medium. Samples were taken at zero, two, three, four, and five generations of growth, and the distribution of labeled DNA fragments in the progeny cells was analyzed by autoradiography. The first segregation between labeled and unlabeled cells occurs at the third generation in both cultures.

The number of labeled cells after three, four, and five generations of growth can be used to calculate the number of labeled pieces, presumably

DNA strands, present after the pulse. The extrapolation indicates that there were four to five (on the average) labeled strands per cell for both BU and thymine labeling. The apparent size of the pieces is similar for both labels; the segregation pattern is identical. The simplest interpretation is that BU has not disrupted the normal replication pattern and is, in fact, incorporated by the normal growing point.

In another type of experiment we examined the replication of individual genes. Exponential cultures of *E. coli* K12 were grown in 5-BU for about one-fourth of a generation. The cells were then infected with the generalized transducing phage P1 in light medium. The resulting lysate was placed on a CsCl gradient, where the hybrid-density transducing particles were separated from the light ones by centrifugation. The transducing activities were then assayed on a multiauxotrophic recipient.

The results show that all markers are replicated to some extent. Since the efficiency of transduction of the markers is diversely affected by BU and other factors, the absolute amount of replication cannot be determined. Essentially, however, the results indicate a normal replication pattern.

The tentative conclusion is that, in our strains of *E. coli* K12, BU is incorporated by the normal replication point and does not induce any major replication aberration. This is in contradistinction to the recently published statement by Abe and Tomizawa[2] that BU induces a new round of replication at the origin.

References

[1]Institut de Biologie Moléculaire, Université de Genève, Switzerland.

[2]M. Abe and J. Tomizawa, *Proc. Natl. Acad. Sci. U.S.* **58**, 1911 (1967).

25.3 EXCISION OF λdg PROPHAGE FROM THE BACTERIAL CHROMOSOME

R. A. Weisberg

λdg's are a class of bacteriophage with the following properties: (1) They are found in low frequency in lysates from induced λ-lysogenic cells; (2) they transduce the galactose (gal) operon of *Escherichia coli;* (3) they do not form plaques because they are missing certain vital phage genes; and (4) the gal operon has replaced the missing phage genes on the phage chromosome. It is generally accepted that λdg's are formed by an abnormal excision of λ prophage from its site on the bacterial chromosome, as shown in Fig. 25.3.1. Once λdg's are formed, they are stable and can be efficiently propagated with the aid of normal λ, which can supply the functions missing in the λdg.

The present work was initiated to test the following hypothesis: that the attachment site of λ, which is necessary for the insertion of the phage chromosome into the proper site on the bacterial chromosome, is homologous to a corresponding attachment site on the bacterial chromosome. If this were true, it would follow that prophage λ is surrounded by two attachment sites (see Fig. 25.3.1), each of which is homologous to the other. Furthermore, it follows that the λdg attachment site is homologous both to the λ attachment site and to the λbio attachment site.

We have tentatively concluded that the λ attachment site cannot be completely homologous to the bacterial attachment site. Our evidence, which follows, suggests that the λdg attachment site is different from the λ and λbio attachment sites:

1. Using the system described last year,[1] we have found that *prophage* λdg does not detach normally (or perhaps not at all) from the bacterial chromosome.

2. If a normal λ prophage or a λbio prophage is adjacent to the λdg prophage, efficient detachment of the λdg prophage (with or without concurrent detachment of the other prophage) can occur.

3. Our evidence indicates that this "help" provided to a λdg by λ or λbio is not due to a gene product, necessary for detachment, which can be formed by the two latter, but not by the former phage, but rather to a structural, cis-acting contribution of the attachment site of the helping prophage. This evidence, which is not as yet conclusive, consists of (1) the inability of λb2, missing part of the λ attachment site, to help and (2) evidence which suggests that, for a superinfecting λ *phage* to help a λdg prophage, the superinfecting phage must first become integrated as prophage.

Reference

[1]R. A. Weisberg, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967,* ORNL-4100, p. 166.

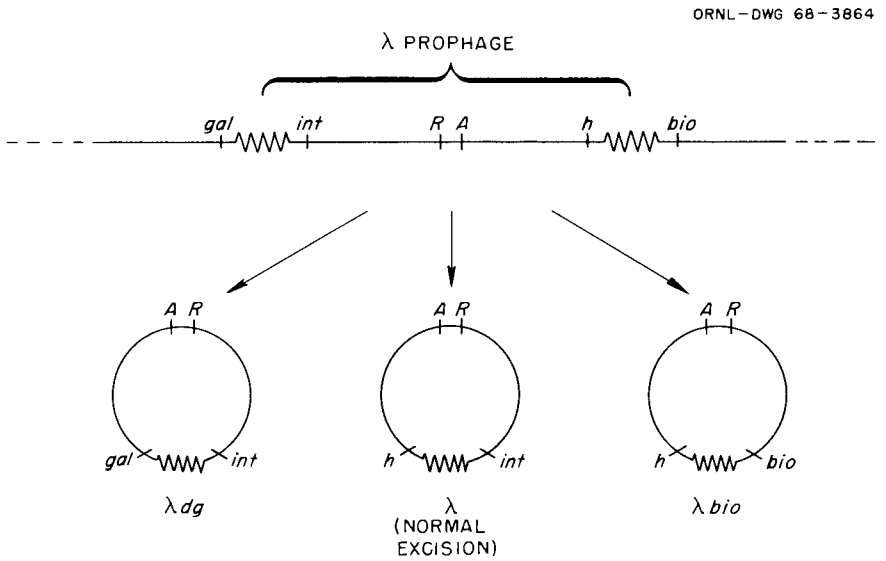

Fig. 25.3.1. Part of the Chromosome of a Lysogenic Bacterium Showing a Normal Excision and Excision of λdg and λbio. *gal* and *bio* are bacterial genes which can be excised with λ; *int*, *R*, *A*, and *h* are λ genes. The straight line represents the bacterial and phoge chromosomes. The zigzag lines represent the λ attachment sites as described by A. Campbell, *Advan. Genet.* 11, 101–37 (1962).

25.4 STUDY OF TRANSDUCTION BY PHAGE P1

Grete Kellenberger-Gujer

In phage P1 lysates, particles are found which contain exclusively bacterial DNA. This host DNA can be transduced to adequate recipient cells and is integrated in their chromosomes. Almost every genetic marker of *E. coli* can be transferred that way with a frequency varying between 10^{-5} and 10^{-4} with respect to the infective P1 particles obtained from the same lysate. If one infects bacteria grown for several generations in heavy medium and transfers them to light medium for the growth cycle of P1, one finds that the transducing particles contain fully heavy DNA.[1] Therefore the bacterial DNA picked up by the phage was already present at the moment of infection and packed into P1 coats without multiplication.

Nothing is known about the sequence of events leading to the encapsulation of DNA into a virus particle, that is, the condensation of the originally extended molecules from the phage DNA pool into a particle of precise shape and size. The lack of synchrony in phage formation and the difficulty of isolating the viral DNA at different stages of maturation do not allow the detection of minor chemical changes in the DNA to be encapsulated. An eventual difference between the DNA of the uninfected cell and the DNA found in the transducing particles, however, could be easily studied. Furthermore, the phenomenon of generalized transduction is in itself certainly worth a more extensive investigation.

A number of approaches were tried:

1. Attempts to isolate a mutant of P1 with a higher frequency of transduction have so far been unsuccessful.

2. Complementation experiments were done between P1 and phage λ to find if P1 produces enzymes promoting transduction or if the uptake of bacterial DNA is linked (and restricted) to P1 structural proteins. No transducing λ particles were found after mixed infection under various conditions. However, a rather strong exclusion of P1 by λ was observed. In spite of the fact that all outcoming λ phages were "modified" by P1 (host-specific modification of DNA), it is possible that other functions of P1 could not be expressed in the mixed infection. The negative result of complementation may, therefore, have other explanations.

3. The effects of transduction of various types of injury to the chromosome of the donor bacteria were studied. Irradiation with x ray or uv over a dose range giving from 100 to 10% survival reduced

the transduction frequency progressively to about 10% of its original value, while P1 multiplication was only very slightly affected. Experiments now in progress are expected to determine whether the uptake of bacterial DNA by the phage, the transfer to the recipient cell, or the integration of the transduced fragment is the affected step. Uv'd HCR^- donors, unable to excise thymine dimers, yielded more transducing particles than HCR^+ bacteria treated with the same doses. Apparently bacterial DNA undergoing repair is less suitable for transduction than DNA carrying uv lesions. A similar conclusion holds for x irradiation also: if one allows the donor to start repair by incubation for 40 min prior to P1 infection, less transducing particles are made than when it is infected immediately after irradiation.

Experiments with very low doses of x ray or uv indicate that neither double-strand breaks nor single-strand excisions promote transduction. This conclusion is based on rough calculations using the data of J. Setlow[2] about the frequency of lesions for a given dose of uv or x irradiation. Surprisingly enough, a short treatment of the donor bacteria with ultrasonic waves increased the transduction frequency markedly.

We plan to extend these studies by testing the influence on transduction of various growth or starvation conditions and that of antibiotics or chemicals suspected to act on DNA.

References

[1]H. Ikeda and J. Tomizawa, *J. Mol. Biol.* 14, 85 (1965); confirmed by my own results.

[2]J. K. Setlow, personal communication.

25.5 SOME PROPERTIES OF ISOLATED PROPLASTIDS FROM ETIOLATED MAIZE LEAVES

Ann B. Jacobson R. W. Williams

Crude plastid preparations from etiolated maize leaves are contaminated with bacteria which come

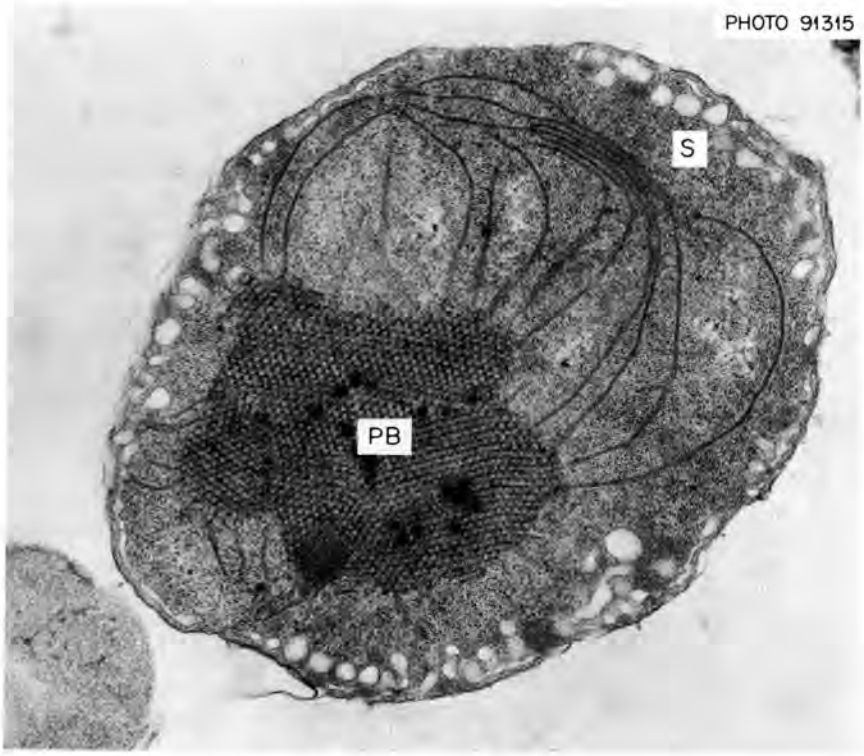

Fig. 25.5.1. An Electron Micrograph Showing a Section Through a Typical Isolated Proplastid from Etiolated Maize Leaves. The organelle appears well preserved. The prolamellar body (PB) is tightly organized; ribosome-like particles are apparent in the stroma (S). 26,000×.

from the leaf surfaces of the plants from which the organelles are isolated. In the past year we have explored techniques for growing sterile plants and have concluded that it is more practical to remove bacteria from plastid preparations during the isolation procedure by repeated low-speed differential centrifugation. The level of contamination in proplastid preparations has been reduced from 13 bacteria per 100 plastids in the initial homogenate to an average level of 7 bacteria per 10^6 plastids in the purified preparations. Because of our recent success in obtaining proplastids which are relatively free of bacterial contaminants, it has been possible to begin experiments on their synthetic properties after isolation.

The isolated proplastid preparations have also been examined with the electron microscope for morphological integrity. As seen in the accompanying electron micrograph (Fig. 25.5.1), the isolated organelles are well preserved. The proplastids have retained their outer membrane, ribosomes are present in the stroma, and prolamellar body is well preserved. These preparations should be quite useful for further studies of proplastid development.

25.6 SEDIMENTATION PROPERTIES OF RIBOSOMAL RNA FROM PROPLASTIDS OF MAIZE

Ann B. Jacobson R. W. Williams

In our previous report[1] we described preliminary results which showed that one component of the ribosomal RNA isolated from proplastids of etiolated

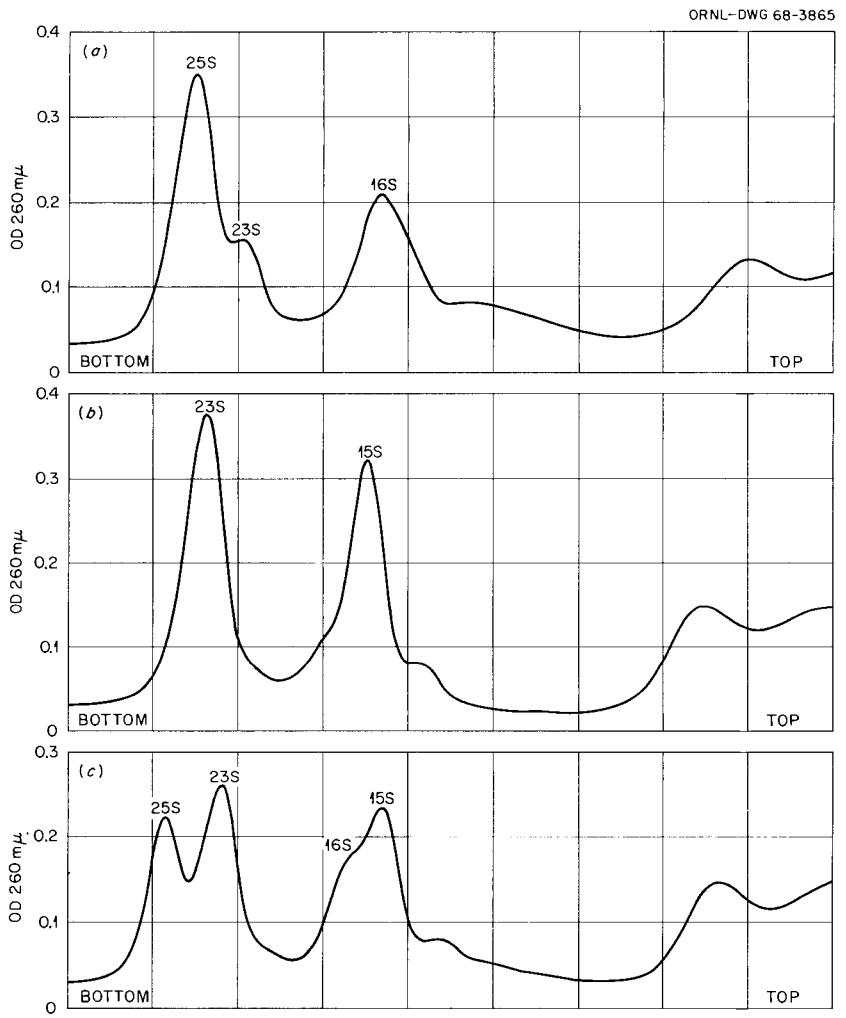

Fig. 25.6.1. A Comparison of Sedimentation Rate of Proplastid and Cytoplasmic RNA on Sucrose Gradients. (a) Whole leaf RNA, (b) plastid RNA, (c) mixture of whole leaf and plastid RNA.

maize leaves was different than the ribosomal RNA isolated from the cytoplasm of the same leaves. During the past year this work has been elaborated. We have employed sucrose gradients capable of greater resolution, and it is now clear that both the heavy and light ribosomal components of plastid RNA sediment at rates different than the cytoplasmic ribosomal RNA's. Approximate sedimentation values have been obtained by calibrating gradients with ribosomal RNA from *Escherichia coli*. We have obtained values of 15 and 23S for plastid ribosomal RNA and 16 and 25S for cytoplasmic ribosomal RNA. These differences are illustrated in Fig. 25.6.1.

Since a unique set of ribosomal RNA's has been identified within plastids, it seems logical that plastid DNA must contain the genetic information which codes for them. Therefore, isolated proplastids should be able to synthesize ribosomal RNA when fed appropriate precursors. Experiments to test this idea are currently under way and should provide some insight into the genetic interaction of nuclear and plastid DNA.

Reference

[1]A. B. Jacobson and P. P. Hester, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, p. 167.

25.7 THE SELECTIVE LOSS OF DNA SATELLITES ON DEPROTEINIZATION WITH PHENOL[1]

Dorothy M. Skinner

The DNA satellite, so-called "d(A-T)" of crabs, is composed of more than 90% alternating deoxyadenylate and deoxythymidylate residues. It makes up from 10 to 30% of the total DNA in the tissues of various species of crabs in the genus *Cancer*.[2] The same "d(A-T)" satellite makes up 18% of the DNA in several tissues of the land crab *Gecarcinus lateralis*.[3] In *Gecarcinus* there is also a second satellite DNA, rich in dC and dG residues, which makes up 3% of the total DNA.

"d(A-T)" is totally lost from DNA isolated from homogenates treated with phenol instead of isoamyl alcohol:chloroform as the protein denaturant. Furthermore, when highly purified DNA preparations containing the three components are treated with hot or cold phenol, both "d(A-T)" and the d(G+C)-rich satellites are lost.[4] The physical characteristics of these satellite DNA's which make them susceptible to phenol are not known. They do not seem to be: (1) molecular size, since sonicated DNA preparations treated with phenol lose all the satellites but retain all main band DNA molecules, even though their average molecular size is smaller than the original size of the satellites; (2) anion used in the extraction; (3) duration or temperature of exposure to high salt and sodium dodecyl sulfate; or (4) protein content, since "d(A-T)" is lost from purified preparations which have less than 1 μg of protein per 25 μg of DNA.

References

[1]Supported in part by USPHS Grant No. AM-06268.

[2]M. Smith, *Biochem. Biophys. Res. Commun.* **10**, 67 (1963).

[3]D. M. Skinner, *Proc. Natl. Acad. Sci. U.S.* **58**, 103 (1967).

[4]D. M. Skinner and L. L. Triplett, *Biochem. Biophys. Res. Commun.* **28**, 892 (1967).

25.8 STUDIES ON THE BIOLOGICAL ROLE OF A DNA SATELLITE

Dorothy M. Skinner

Wallace and Bernsteil[1] showed that the fraction of DNA in *Xenopus* which is complementary to ribosomal RNA (rRNA) is G+C-rich, having a density in CsCl of 1.723. This density is the same as that of the d(G+C)-rich satellite in *Gecarcinus lateralis* DNA. Therefore we have compared the hybridization of ^{32}P-labeled rRNA from midgut gland both with unfractionated DNA containing the d(G+C)-rich satellite and with purified main band DNA. We find that land crab rRNA is complementary to 3.5 to 4.5% of the total DNA and 1.8% of the main band DNA. The possibility that the latter is due to contaminations of the main band DNA with the d(G+C)-rich satellite has not yet been ruled out. In any event, complementarity of 3 to 4% is more than ten times the amount of rRNA-coding DNA found in adult vertebrates and is possibly related to the fact that rRNA appears to be synthesized in bursts at the onset of the pre-

molt period[2] and that a high redundancy of genes coding for rRNA is needed to support this burst of synthetic activity. If this hypothesis is correct, a high fraction of DNA coding for rRNA should be found in all those invertebrates whose ribosome- and protein-synthesizing activity is restricted to a brief fraction of their life cycle.

References

[1]H. Wallace and M. L. Bernsteil, *Biochim. Biophys. Acta* **114**, 296 (1966).
[2]D. M. Skinner, *Am. Zool.* **6**, 235 (1966).

25.9 DETERMINATION OF THE MOLECULAR WEIGHT OF MITOCHONDRIAL DNA IN *PHYSARUM POLYCEPHALUM*

R. A. McGrath R. W. Williams

The molecular weight of mitochondrial DNA from the slime mold *Physarum* was determined as follows: Cultures having been grown for several generations in the presence of ^{3}H-thymidine and mitochondria were isolated, essentially by the procedure of T. Evans.[1] Samples of the ^{3}H-labeled mitochondria were then centrifuged in alkaline sucrose gradients (5 to 20% sucrose at pH 12.0, 20°C, 3.0 hr at 30,000 rpm in an SW 39 rotor). Contents of centrifuge tubes were collected as two-drop fractions on filter paper disks. Disks were processed to remove acid-soluble radioactivity, and the acid-insoluble fraction was detected by liquid scintillation counting.

Pieces of single-stranded DNA, isolated as outlined above, have a molecular weight of approximately 8×10^6 daltons. For comparative purposes we have found, using similar methods, other molecular weights as follows: Slime mold (*Physarum*) nuclear DNA, 4×10^7 daltons; *E. coli* DNA, 3×10^8 daltons; T-2 bacteriophage DNA, 6×10^7 daltons.

Mitochondria were biochemically active, following the isolation procedure, as evidenced by continued succinic dehydrogenase activity, but we have been unable as yet to rule out the possibility that some degradation of DNA may have occurred during isolation. For this reason, the molecular weight given for mitochondrial DNA must be considered as a minimum average value.

Reference

[1]Western Reserve University, Cleveland, Ohio.

25.10 USE OF CHEMICAL ^{3}H-METHYLATION IN THE DETERMINATION OF SEDIMENTATION COEFFICIENTS OF DNA IN ALKALINE SUCROSE

J. S. Cook A. J. Zelnis

We have developed a method for determining, in alkaline sucrose, the sedimentation coefficients of large pieces of single-stranded DNA from organisms whose DNA cannot be readily labeled in vivo. The method is based on the alkaline sucrose gradient technique of McGrath and Williams,[1] in which cells or cell organelles are introduced into a thin layer of 0.5 to 1.0 *N* NaOH overlaying an alkaline sucrose gradient (5 to 20% sucrose, pH 12). After appropriate time has been allowed for lysis of the biological material, the single-stranded DNA is centrifuged, collected, and analyzed for acid-insoluble radioactivity.

Our extension of the method has been to overlay the NaOH layer, containing lysed cells or organelles, with ^{3}H-dimethyl sulfate dissolved in benzene. The benzene is necessary, since otherwise the dimethyl sulfate ($\rho = 1.332$) sinks to the bottom of the gradient. As the benzene (bp = 80°C) is evaporated by a gentle air stream, the dimethyl sulfate (bp = 188°C) goes into the aqueous phase, where, under the prevailing alkaline conditions, the DNA is methylated and so ^{3}H-labeled. Since other trichloroacetic acid-insoluble molecules are also methylated, considerable TCA-insoluble radioactivity may be found at the top of such gradients. Nevertheless, the DNA molecules are of such a relatively large size that they may be readily separated from other methylated components. In control experiments with ^{14}C-labeled T4 bacteriophage, there was no evidence that under these conditions the ^{3}H-methylation affected the sedimentation properties of the DNA.

The method appears suitable for determination of sedimentation coefficients of DNA from *Neurospora*, higher plants, and other organisms or organelles in which the DNA is not readily labeled in vivo.

Reference

[1]R. A. McGrath and R. W. Williams, *Nature* **212**, 534 (1966).

25.11 PHOTOREACTIVATION AND PHOTOREACTIVATING ENZYME IN EUKARYOTIC CELLS

J. S. Cook Janet R. McGrath

In our studies on the biology of photoreactivation and the cellular functions of photoreactivating enzyme, we have investigated the distribution of the enzyme activity in frog liver, a vertebrate tissue with a comparatively high level of such activity.[1] Enzyme activity was assayed by the ability of extracts of cell fractions to photoreactivate, in vitro, uv-irradiated transforming DNA from *Hemophilus influenzae*.[2] We find that when the cells are gently homogenized in sucrose-$CaCl_2$, Tris-buffered to pH 8.0, 80% of the total activity is associated with the nuclear fraction and the remaining 20% is in the soluble fraction. No activity (i.e., less than 3% of the total) was detectable in the mitochondria-rich fraction. The activity in the nuclear fraction is very loosely bound and is lost to the soluble fraction if the tissue is homogenized in isotonic KCl. This fact led to our original tentative conclusions,[3] derived from preliminary experiments, that the activity was all in the soluble fraction. It now appears, on the contrary, that even the 20% of the total which we find in the soluble fraction might in fact be released from nuclei during preparation.

Although we were not able to demonstrate photoreactivating-enzyme activity in frog liver mitochondria, there exist several biological systems in which photoreactivation of mitochondrion-associated functions has been described. In order to investigate directly the chemical nature of the phenomenon in such systems, in many of which the usual radioactive labeling techniques are not satisfactory, we are presently developing new methods for labeling DNA photoproducts in DNA isolated from cells after experimental treatment.

References

[1] J. S. Cook and J. R. McGrath, *Proc. Natl. Acad. Sci. U.S.* **58**, 1359 (1967).

[2] A. Muhammed, *J. Biol. Chem.* **241**, 516 (1966).

[3] J. S. Cook and J. R. McGrath, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, pp. 172–73.

25.12 THE EXISTENCE OF CYCLOBUTANE PYRIMIDINE DIMERS IN DNA

R. B. Setlow W. L. Carrier

A recent paper[1] questions the existence of dimers in DNA after ultraviolet irradiation, because the fraction of the total radioactivity in dimers labeled with ^{3}H-methyl thymine was found to be about 30% less than the fraction of dimers labeled with 2-^{14}C-thymine. We have attempted to repeat these observations but have not been able to do so. Figure 25.12.1 shows one of a

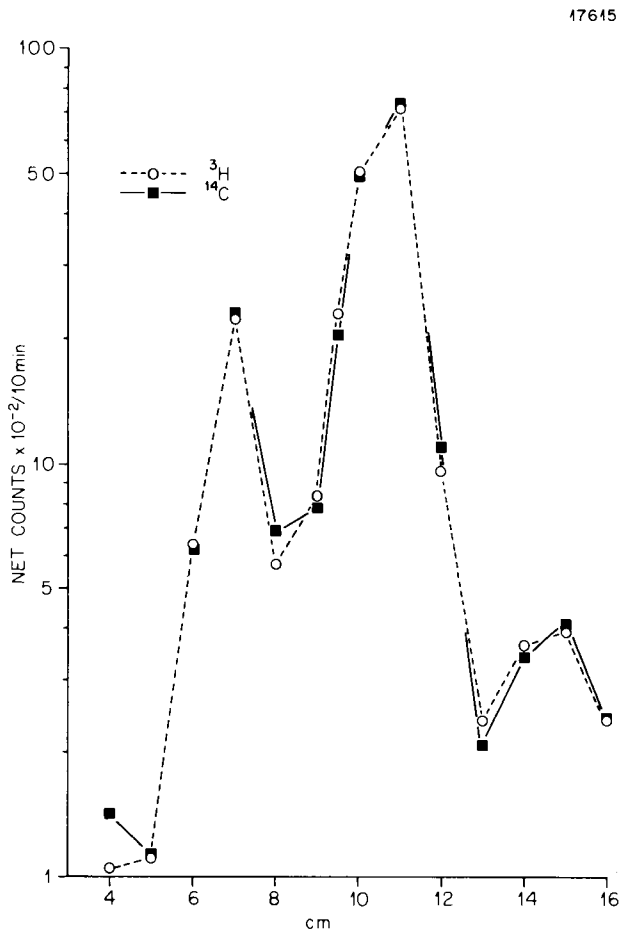

Fig. 25.12.1. The Photoproduct Section of a Radiochromatogram of a Hydrolysate of an Irradiated *E. coli* DNA Labeled with Both ^{3}H- and ^{14}C-Thymine. The radioactivity under the peaks is the same for both ^{3}H and ^{14}C. The barely perceptible difference in chromatographic mobilities of ^{3}H and ^{14}C activities is an isotope effect. The products at 7, 11, and 15 cm are $\hat{UT}$, $\hat{TT}$, and unknown respectively.

series of five experiments on the irradiation in vivo of DNA labeled with both ^3H and ^{14}C, using doses from 200 to 4×10^4 ergs/mm^2. We observed that the ^3H and ^{14}C dimers had the same activity ±2% mean deviation. Wang *et al.* have made a systematic error in their procedures for the simultaneous counting of ^3H and ^{14}C in a scintillation counter. All present evidence indicates that dimers are not artifacts of preparative procedures but do indeed exist in DNA.

Reference

[1]S. Y. Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.* **57**, 465 (1967).

25.13 THE EXCISION OF PYRIMIDINE DIMERS IN VIVO AND IN VITRO

R. B. Setlow W. L. Carrier

The ultraviolet irradiation of DNA in vivo or in vitro usually results in the formation of cyclobutane-type dimers between adjacent pyrimidine residues in the polynucleotide chains. Because such dimers (1) have biological consequences, (2) are stable to acid hydrolysis, and (3) are easy to detect in small numbers by radioactive labeling and chromatographic procedures, we have used dimers as models in investigations of the repair of DNA. In some strains of bacteria (usually uv sensitive) the dimers remain in DNA; in others (usually uv resistant) the dimers are removed selectively from the bacterial DNA as parts of oligonucleotides. The latter process, termed excision, is associated in vivo with the well-coordinated steps of repair replication and the rejoining of strand breaks that result from excision. Obviously the biological effectiveness of these processes depends on their rates compared with DNA synthesis "around" a lesion or past a strand break. We have investigated three excision systems – *E. coli*, T4 phage, and extracts of *M. lysodeikticus*.

In *E. coli* excision is inhibited by caffeine, the acridine dyes, KCN, and the absence of a carbon source, but not by thymine deprivation in thymine-requiring strains or by chloramphenicol. Thus excision is an energy-dependent process but does not require normal DNA replication or protein synthesis. During the excision of dimers relatively few breaks per chromosome are observed, and if the excision rate is inhibited the number of breaks does *not* increase. The *E. coli* system does not work on injected T4 DNA.

In cells infected with T4v phage the excision activity is observed in both uv-sensitive and -resistant cells. It acts soon after phage infection on T4v, T4v$^-$, and bacterial DNA. The activity does not appear in starved cells or in cells infected in the presence of KCN or very large amounts of chloramphenicol.

Extracts of *M. lysodeikticus* contain nuclease activities that excise dimers from uv-irradiated DNA without extensive degradation of the DNA.[1] Excision requires the sequential action of two components of the extract.[2] One of the nucleases involved in the excision of dimers has been shown to be an endonuclease specific for uv-damaged DNA. One of the problems in its purification is the measurement of its enzymic activity by simple means. We are attempting to do this by using small Sepharose columns to separate DNA (molecular weight $> 10^6$) from the polynucleotides (molecular weight $\sim 10^5$) that result from endonucleolytic attack on uv-irradiated DNA. If reaction mixtures are run with ^3H-labeled uv DNA and ^{14}C-labeled unirradiated DNA, the ratio of ^3H to ^{14}C in the void volume gives a fast and reliable assay of the uv-specific endonuclease activity.

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25.14 PHOTOREACTIVATION IN VIVO OF THE DNA OF T4 PHAGE

R. B. Setlow W. L. Carrier

Pyrimidine dimers of the cyclobutane type are monomerized by photoreactivating (PR) enzyme plus near-uv or visible light. Hence the fraction of uv-induced biological damage that can be reversed by photoreactivation (the PR sector) is a measure of the fraction of biological damage that arises from dimers. In practice the PR sector has been taken to be the maximum fraction of damage

due to dimers because biological PR could arise by indirect means. The PR sector for T4v$^-$ phage is ~0.5. We tried to determine if all the dimers were monomerized when the phages were maximally photoreactivated.

T4v$^-$ phages (nonexcising) were labeled with ^{3}H-thymine and irradiated with 265-nm radiation. The phages were adsorbed at multiplicities of infection between 0.2 and 5 to *E. coli* B_{s-8} or B/r and exposed to 405- or 365-nm radiation (for photoreactivation) under conditions (starvation or 0.01 *M* KCN) in which there is no phage development or dimer excision. Samples were analyzed for plaque-forming ability and dimers.

The dose reduction factors were the same for both plaque formation and dimer destruction. About 60% of the dimers in the phage DNA injected into host cells were *not* monomerizable, whereas, under similar conditions, over 80% of the dimers induced in bacterial DNA were destroyed. Thus dimers in phage DNA are not all equally accessible to photoreactivating enzyme in vivo.[1] In our experiments the fraction of damage ascribable to dimers in T4v$^-$ phage may be close to 1.0 rather than the value 0.5 derived from photoreactivation of plaque-forming ability.

Reference

[1]In collaboration with J. K. Setlow we have shown that dimers in phage DNA are destroyed by PR treatment in vitro at about the same rate as those in bacterial DNA.

25.15 PYRIMIDINE DIMERS IN *PARAMECIUM* DNA

Betsy M. Sutherland[1] W. L. Carrier
R. B. Setlow

It has not been possible to evaluate the role of pyrimidine dimers in uv-induced mutation in higher organisms, since dimers and their fate and mutation production have not been investigated in the same eukaryote. *Paramecium aurelia*, a ciliate protozoan, is known to be amenable to mutation studies. Therefore, we have used *Paramecium* as the subject of three experimental series: the production of thymine-containing dimers in the range of biological doses, the fate of dimers in cells incubated in the dark after irradiation, and the "loss of photoreactivability" of dimers with time after irradiation. These experiments, together with data on photoreactivation in *Paramecium*, support the hypothesis that pyrimidine dimers in the DNA of *P. aurelia* play an important role in induction of mutations by ultraviolet radiation.

The paramecia were labeled with ^{3}H-thymidine by letting them feed on *E. coli* cells that had been previously labeled with this DNA precursor.[2] They were then transferred to a uv-transparent salt solution for irradiation with 2537-A radiation. After irradiation the animals were returned to the normal growth conditions and analyzed at various times for thymine-containing pyrimidine dimers. Although bacteria were present in the labeling system, the photoproducts (dimers) were identified as in paramecium DNA on the basis of experiments using autoradiography of labeled animals, cesium chloride density gradients of extracted DNA, and reirradiation of the photoproducts by 2390-A radiation.

Exposure of cells to 4000 ergs/mm^2 of uv results in the formation of pyrimidine dimers that contain ~0.25% of the thymine radioactivity in paramecium DNA. (This number is appreciably smaller than is observed for DNA irradiated in vitro because of the strong absorption of the incident radiation by the cytoplasm.) The further irradiation of these cells with black light destroys the dimers.[3] This is the first demonstration of in vivo photoreactivation of pyrimidine dimers in a eukaryote. If uv-irradiated cells are incubated in the dark, the dimer content of the DNA does not change for about 6 hr, then decreases to 0 at about 21 hr. The dimers do not appear in the small-molecular-weight fraction of the cells and are probably expelled into the medium.

These data may be compared with three series of mutation experiments: First, both dimers and mutations are produced in the same range of doses. Second, photoreactivating light exposures of the same order of magnitude reduce dimers and mutation to the level of the unirradiated controls. Third, the removal of dimers from the DNA in the dark could be a step in the repair of premutational lesions.

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25.16 PHOTOREACTIVATION, PHOTOPROTECTION, AND PHOTOINACTIVATION OF INDUCED FORMATION OF ENZYMES IN *ESCHERICHIA COLI* B/r

P. A. Swenson

Ultraviolet (uv) radiations inhibit the induced formation of β-galactosidase and tryptophanase. When the output from a G-E "black" lamp (main emission at 365 nm) is used as photoreactivating light, considerable photoreactivation of the uv-inactivated β-galactosidase system is obtained. The photoreactivation effect is observed immediately after induction. Photoreactivation is also obtained using monochromatic radiation at 405 nm. No photoreactivation of the system is obtained with *E. coli* H/r, a strain which does not possess a photoreactivating enzyme. These data show that photoreactivation of β-galactosidase involves monomerization of pyrimidine dimers.

Photoprotection is a process which is temperature independent. *E. coli* B/r cells were illuminated at 5°C for 20 min using the black lamp, and control cells were stirred at 5°C. Both suspensions were then treated with uv at room temperature, and marked photoprotection was obtained. However, the uv-irradiated cells held at 5°C without black light formed β-galactosidase at a much higher initial rate than cells irradiated and induced before the holding treatment at 5°C. The black lamp treatment alone at 5°C had only a slight inhibitory effect, but holding the cells at 5°C without black light or uv had no stimulatory or inhibitory effect.

Experiments were also carried out in which the B/r cells were cooled quickly to 5°C, exposed to uv radiations at that temperature, and then warmed to 37°C and induced. This temperature treatment gave the same protection to uv as did the cooling of the cells for 20 min before uv was given at room temperature. Neither of the cold treatments would be expected to affect the configuration of DNA and cause a difference in the numbers or types of uv photoproducts. A possible explanation is that the low temperature releases the cell from catabolite repression. Catabolite repression refers to the fact that the β-galactosidase system is more sensitive to uv when glycerol is present than when absent.

Preliminary work has already shown that photoreactivating treatment with the black light at room temperature does not photoreactivate the tryptophanase system immediately; incubation of the cells for 30 min at 37°C produces a delayed photoreactivation. The explanation is provided by the results of an experiment showing that the black lamp treatment alone inhibits tryptophanase formation by 80% for about 30 min. After this inhibitory period, recovery occurs. Inhibition by the black lamp treatment is also effective at 5°C, indicating that a photochemical process not associated with enzymatic reactions is involved in this inhibition. The strong photoinactivation by near-uv radiation of the tryptophanase-forming system but not of the β-galactosidase-forming system indicates that action spectrum studies in the near-uv region may help in the understanding of the process of tryptophanase induction.

25.17 INHIBITION OF β-GALACTOSIDASE SYNTHESIS BY UV IN *ESCHERICHIA COLI* B IN THE ABSENCE OF CATABOLITE REPRESSION

P. A. Swenson

Formation of the induced enzyme β-galactosidase in *E. coli* is known to be more sensitive to uv when glycerol is present (conditions of catabolite repression) than when glycerol is removed from the medium prior to irradiation. Catabolite repression might account for the fact that when β-galactosidase synthesis is partially inhibited by uv in *E. coli* B_{s-1} (a radiation-sensitive strain) the cells lose subsequently over a 90-min period the ability to form the enzyme. Time-course studies of β-galactosidase formation have been carried out using *E. coli* B_{s-1} and B/r (a radiation-resistant strain) in the presence and absence of glycerol and with and without radiation. Concurrent studies of DNA synthesis, growth, and respiration were also made. Catabolite repression has been ruled out as the cause of the progressive loss of β-galactosidase-forming ability in B_{s-1}. The data indicate that the loss is part of a process in which all cellular synthesis is gradually un-

coupled from respiration. These experiments show the consequences of a small number of uv-induced lesions in cells that do not have repair systems – consequences that lead to the death of the cell.

25.18 THE PHYSIOLOGY OF UV-IRRADIATED *ESCHERICHIA COLI*

Barbara A. Hamkalo P. A. Swenson

Growth and respiration become uncoupled in uv-irradiated log-phase cultures of *E. coli* B_{s-1}, a strain unable to repair uv-damaged DNA in the dark. In a strain with the ability to repair (*E. coli* B/r), these processes remain closely coupled, except at extremely high uv doses. At a dose of 400 ergs/mm^2, respiration and growth responses of B/r after uv vary with various carbon sources upon which cells are grown and incubated after irradiation. Succinate- or glycerol-grown cells show severe respiratory inhibition within 60 min that persists for a dose-dependent period of time. Acetate-grown cells show less severe inhibition, pyruvate-grown cells show slight inhibition for a dose-dependent period, and glucose-grown cells show a lowering of the respiratory rate, beginning immediately after uv, this rate never being severely inhibited.

The different responses with various carbon sources indicate the existence of alternate metabolic pathways that may respond differently to radiation. Experiments in which the carbon source is changed immediately after uv provide evidence for the existence of such pathways. Cells grown on glycerol, washed and irradiated in buffer, then given pyruvate or glucose as energy sources do not respond like cells grown on these substrates (little respiratory inhibition). The cells do show a response similar to cells that have been grown and irradiated in glycerol medium (severe respiratory inhibition for a time). We interpret these results as evidence for the oxidation of glucose and pyruvate after irradiation by pathways common to glucose, pyruvate, and glycerol. The enzymes of alternate pathways may be inducible only in the presence of substrate. Since uv inhibits enzyme induction,[1] such enzymes would not be present in cells until DNA synthesis resumes and inducibility is regained.

Pyruvate in the suspending medium at the time of uv irradiation has been reported as an extremely effective protective agent.[2] We have found that pyruvate solutions at the concentrations reported absorb uv radiation completely. Cells grown on pyruvate but irradiated in buffer show the same sensitivity to killing by uv as cells grown on glycerol.

In B/r, the severe inhibition of respiration of glycerol-grown cells can be photoreactivated (PR) with either black light (330 to 400 nm) or monochromatic light at 405 nm. The latter wavelength is effective only in direct PR (monomerization of pyrimidine dimers). We may also rule out indirect PR because the inhibition cannot be photoreactivated in *E. coli* H/r, a radiation-resistant strain which does not possess the photoreactivating enzyme. Chemical measurements of total DNA, RNA, and protein can be correlated with the respiratory response of irradiated glycerol-grown cells. Immediately after a uv dose of 400 ergs/mm^2, total DNA is constant for $\sim$1 hr, while there is considerable increase in total RNA and protein. During the period of severe respiratory inhibition, there is very little increase in total RNA and protein, but DNA has begun to increase. Later, when respiration resumes, RNA and protein synthesis also resume.

The inhibition of DNA synthesis resulting from uv-induced pyrimidine dimers in DNA probably results in an unbalanced metabolic state in cells: an accumulation of protein and RNA synthesized during the period of DNA synthesis inhibition. Metabolic controls maintaining constant quantitative relationships among these macromolecules in unirradiated cells may function to reestablish metabolic equilibrium in cells which possess the ability to repair uv damage. The expression of this control may vary with the oxidative pathway utilized.

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25.19 ATP LEVELS IN UV-IRRADIATED *ESCHERICHIA COLI* B/r

Barbara A. Hamkalo P. A. Swenson
J. W. Longworth

DNA synthesis is inhibited in *Escherichia coli* B/r immediately after 254-nm irradiation. Since

this strain possesses the ability to repair uv-induced lesions in DNA, DNA synthesis resumes after a lag period. Four hundred ergs/mm^2 induces a 60-min lag. If cells are grown and irradiated in glycerol medium, one observes severe inhibition of respiration setting in at about 60 min and persisting for about 150 min. Because ATP is required for DNA synthesis, we were concerned with the lack of oxidative activity and phosphorylation while DNA synthesis was resuming.

In order to resolve this paradox, we have measured total ATP in sonicated extracts of uv-irradiated *E. coli* B/r by the luciferin-luciferase assay.[1] Within 30 min after uv, irradiated cells contain three to four times the normal ATP per unit cell mass. This excess value slowly drops to the unirradiated control value. It has been reported that x-irradiated *E. coli* leak increasing amounts of ATP into the medium with time.[2] The amounts of leakage in uv-irradiated cells depend on the energy source being oxidized, but leakage doesn't exceed ~20% under most conditions. Succinate-grown cells are unique, since they leak up to 50% of the ATP accumulated. The rapid accumulation of ATP immediately after uv may be the result of the inhibition of DNA synthesis. The ATP is largely retained by the cells and would be available both as a precursor of dATP and as an energy source for polymerization when DNA synthesis resumes.

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25.20 EVIDENCE FOR TWO KINDS OF BASE STACKING IN POLY rA

R. O. Rahn J. W. Longworth
R. I. Fox

We are interested in correlating the luminescence properties of polynucleotides with observed conformational changes in the polymer. Poly rA exists in neutral buffer as a single-stranded stacked helix whose emission properties are similar to those of the free adenine bases. On the other hand, poly dA has a red-shifted fluorescence and quenched phosphorescence relative to the free bases. Poly rA and poly dA are known to have the same degree of stacking interaction, but their optical rotary dispersion (ORD) patterns are vastly different. Hence, the nature of the stacking in these two polymers is not the same. We have found that the nature of the stacking in poly rA can be changed by the addition of equimolar amounts of divalent metal ions, provided there is a denaturant present such as ethylene glycol. The resulting ORD patterns and luminescence spectra of the poly-rA–metal-ion complex resemble very strongly those obtained for poly dA.

Optical absorbance and ORD measurements were made on poly rA either in neutral buffer or in a 50% ethylene glycol–buffer mixture. The glycol disrupted the base stacking, as judged by the uv hyperchromism. There was also a decrease in the optical rotation. Divalent metal ions such as Ca^{2+} or Mg^{2+} restored base stacking but led to an even further decrease in the optical rotation. The resulting ORD pattern resembled that of poly dA. Divalent metal ions in the absence of glycol had no effect on the poly rA structure.

It has been proposed[1] that in poly rA the 2′-OH group is involved in hydrogen bonding with the base. As a result the bases are less in parallel than in poly dA, and the rotational strength is greater than for poly dA, in accordance with exciton theory. The intramolecular hydrogen bonding in poly rA is destroyed by glycol. Divalent metal ions cause the bases to restack but presumably in a more parallel fashion, like that found in poly dA.

Reference

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25.21 AGGREGATION OF SINGLE-STRANDED DNA WITH Mg^{2+}

R. O. Rahn

It has been claimed[1] that Mg^{2+} binds to denatured DNA at temperatures above the melting point in such a way that upon cooling the metal-ion–DNA complex shows a strong red-shifted absorption spectrum. Addition of Mg^{2+} to denatured DNA at room temperature does not cause any such

shift in the absorption spectrum. We have found that these observations can be explained by aggregation of the single-stranded DNA by Mg^{2+} and not by the proposal that preferential binding of Mg^{2+} to the bases occurs at high temperatures.

Calf-thymus DNA in buffer was heat denatured with Mg^{2+} (0.04 *M*) present. Upon cooling, the absorption spectrum was measured and found to have an apparent shift to longer wavelengths. However, low-speed centrifugation removed completely all absorbing material. Hence the absorbance shift is due only to the aggregation of the DNA. In buffer, heat-denatured DNA renatures sufficiently to inhibit aggregation upon addition of Mg^{2+} at 25°C. However, if glycol, which is a denaturant and prevents both stacking and strands from reforming, is present, then addition of Mg^{2+} at 25°C leads to aggregation.

Mg^{2+} aggregates single-strand unstacked DNA but not stacked or partially renatured DNA.

Reference

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25.22 INFLUENCE OF BASE STACKING ON THYMINE DIMER FORMATION IN DNA: SOLVENT AND TEMPERATURE PERTURBATIONS

R. O. Rahn J. L. Hosszu

It has been proposed that base stacking favors thymine dimer formation in TpT.[1,2] In order to determine whether base stacking favors dimer formation in DNA, we measured the thymine dimer yield in both native and denatured DNA as a function of the irradiation temperature and of the solvent. In the absence of denaturing agents and at low temperatures, the bases in both native and denatured DNA are highly stacked. By either raising the temperature and/or adding a denaturant, we varied the degree of base stacking in DNA and correlated it with the observed changes in dimer yield.

Thymine-containing dimers were measured by counting the radioactivity contained in the dimers isolated from hydrolyzed ^{3}H-labeled *E. coli* DNA. The dimer yield for native DNA in buffer as a function of temperature showed a very gradual increase with increasing temperature up to the melting point T_m. Upon passing through the melting temperature, the yield decreased sharply, corresponding to the change from native, stacked DNA to denatured, unstacked DNA. In heat-denatured DNA in buffer, the dimer yield, which was 30% greater than that in native DNA at 25°C, decreased linearly with increasing temperature and joined the curve obtained for native DNA above T_m.

Addition of ethylene glycol to denatured DNA destroyed the base stacking as determined by absorbance measurements and gave lower dimer yields. In native DNA, the addition of glycol only decreased T_m and did not change the yield.

The yield of thymine dimer in *native* DNA is relatively insensitive to changes in both the solvent and the temperature (between 20°C and T_m). The loss of base stacking, which occurs only after disruption of the double-stranded form, leads to lower dimer yields.

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25.23 LOW-TEMPERATURE PHOTOCHEMISTRY AND PHOTOBIOLOGY OF DNA

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J. L. Hosszu

In order to assess the relative importance of different uv photoproducts in the biological inactivation of transforming DNA, it is necessary to have some means of altering the relative amount of the different products. We have used two methods of changing the relative photoproduct concentrations: (1) varying the temperature of uv irradiation and (2) photoreactivating the transforming DNA with yeast photoreactivating enzyme, which monomerizes pyrimidine dimers. We have measured three different types of photoproducts and the biological inactivation of *Hemophilus influenzae* transforming DNA irradiated at a range of temperatures from 25 down to −180°C, before and after maximum photoreactivation. These photoproducts were: (1)

thymine-containing dimers, (2) another thymine-containing photoproduct[1] (spore-type, SP), which, unlike the dimers, is formed in purified DNA only at low temperatures, and (3) the cross link.

Thymine-containing dimers and SP were measured by counting the radioactivity in dimers separated from hydrolyzed tritium-containing DNA. Cross links were measured by their ability to prevent irreversible absorbance changes due to strand separation following heat denaturation of the DNA.

The biological inactivation and the dimer formation at the various temperatures show a clear correlation and in general decrease with decreasing temperature. Photoreactivation, which eliminates the dimers, does not alter the cross links or SP. The biological inactivation after maximum photoreactivation is greatest at −100°C, which is the same temperature maximum found for SP formation. The formation of cross links decreases with decreasing temperatures below 25°C.

Pyrimidine dimers account for a significant fraction of the biological damage even at low temperatures, where their relative contribution is smaller. SP probably accounts for some but not all of the nonphotoreactivable damage at low temperatures. Cross links could be responsible for some of the nonphotoreactivable damage after large doses at room temperature but do not contribute appreciably to low-temperature inactivation.

Reference

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25.24 PHOTOCHEMISTRY AND PHOTOBIOLOGY OF BACTERIAL SPORES: TEMPERATURE DEPENDENCE

J. E. Donnellan, Jr. R. O. Rahn
J. L. Hosszu R. S. Stafford

Bacterial spores irradiated with ultraviolet light at liquid-air temperature were 15 to 20 times more resistant than those irradiated at room temperature,[1] while spores irradiated at −79°C were 2.5 times more sensitive.[2] When pure DNA is irradiated in the frozen state, a photoproduct similar to the photoproduct formed in spores irradiated at room temperature is formed.[3] The production of this spore-type photoproduct in frozen DNA was maximal around −80°C, less at −196°C, and nonexistent at room temperature. We have studied the photochemistry and photobiology of *Bacillus megaterium* spores irradiated at a range of temperatures between 26 and −196°C.

Spores of *B. megaterium* strain QM B1551 labeled with ^{3}H-thymidine were exposed to a constant dose of uv in an adjustable temperature apparatus. The samples were then tested for the colony-forming ability of the spores and the amount of thymine photoproduct in the spore DNA. Our results on the survival of colony-forming ability are similar to previous findings in that the spores were more sensitive at −90°C than at 26°C, while they were extremely resistant at −196°C. The survival in between these extremes was intermediate. The rate of photoproduct production also exhibited a maximum at −90°C; in fact, the functional dependence with temperature was identical to that of survival. No new photoproducts were observed in these experiments.

These experiments support the hypothesis that the thymine photoproducts produced by uv irradiation of bacterial spores are involved in the mechanism leading to spore death. The production of a new spore-type photoproduct in frozen DNA and the DNA of vegetative cells arises presumably from a conformation change in the DNA. The DNA in spores probably does not undergo a conformation change upon freezing, since no new photoproducts were observed in these experiments.

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25.25 THE ULTRAVIOLET PHOTOCHEMISTRY OF GERMINATING SPORES

R. S. Stafford J. E. Donnellan, Jr.

Ultraviolet (uv) irradiation of bacterial spores produces thymine-containing photoproducts different from those produced in vegetative cells. This can explain the extreme uv resistance of bacterial spores. Germinating bacterial spores,

however, exhibit an even more extreme resistance to ultraviolet irradiation.[1] Our previous studies with *Bacillus subtilis* spores suggested that the resistance was attributable to a decrease in the production by uv of thymine-containing photoproducts.[2] We have confirmed this result using *Bacillus megaterium* spores, which germinate more synchronously than those of *B. subtilis*.

Spores of *B. megaterium* QM B1551 were produced in a liver extract medium containing ^{3}H-thymidine. After removal from the spent sporulation medium, the spores were placed in germination medium to start the germination process. At various times aliquots were removed and subjected to a single dose of uv radiation (3200 ergs/mm^2). The aliquots were then analyzed for survival of colony-forming ability and for radioactive photoproducts.

Figure 25.25.1 shows the results of these experiments. The peak survival, that is, highest resistance, is reached after about 3 min germination and achieves a maximum of 55% (Fig. 25.25.1*A*). After this exposure, dormant spores exhibited 0.25% survival, whereas log-phase vegetative cell survival would have been less than 0.01%. During the time uv resistance is increasing, the spore-type photoproducts decrease rapidly, while vegetative cell-type photoproducts (cyclobutane-type thymine dimers) are not produced at all; in fact, the radioactivity in the thymine dimer region also decreases (Fig. 25.25.1*B*). Finally, as the uv survival starts to decrease, the production of cyclobutane-type dimers commences and eventually reaches levels typical of vegetative cells. Thus when uv survival is at a maximum (3 min after the start of germination), the total amount of thymine photoproduct is at a minimum and is an order of magnitude less than the levels found in dormant spores or vegetative cells.

From stereochemical studies, J. W. Longworth has suggested that the cyclobutane-type dimer cannot form in the normal hydrogen-bonded B form

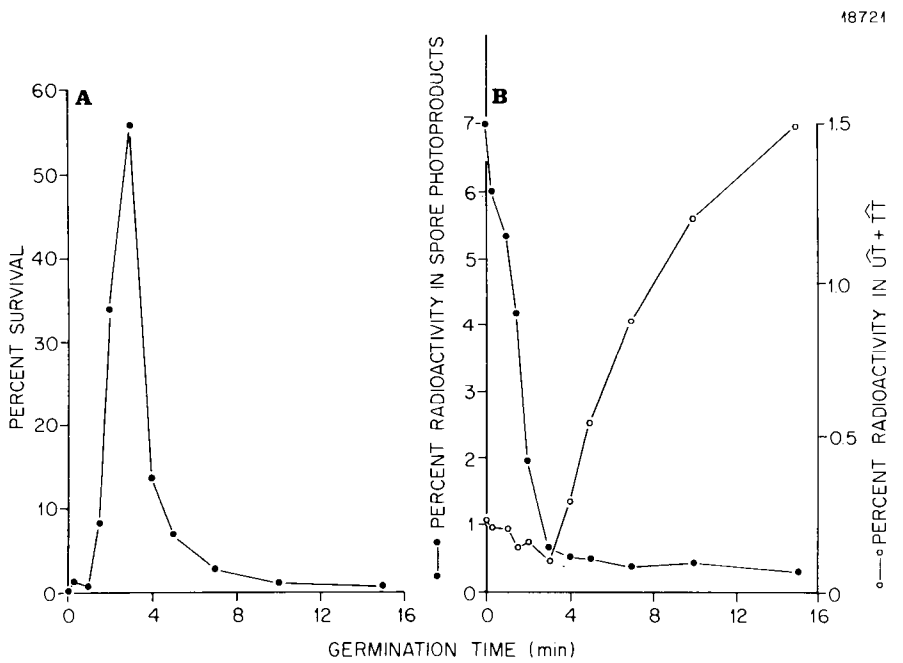

Fig. 25.25.1. (*A*) Survival of *B. megaterium* Spores and Germinating Spores as a Function of Germination Time. Spores were placed in germination medium and allowed to differentiate for various times before being exposed to a constant 20-sec exposure of 3200 ergs/mm^2 at 2650 A. Zero-time sample represents survival of dormant spores irradiated in water. (*B*) Percent of Thymine in Photoproducts in Germinating Spores of *B. megaterium* as a Function of Germination Time. Portions of the samples used to determine the survival of germinating spores to a dose of 3200 ergs/mm^2 in *A* were analyzed for spore photoproducts (SP) and total thymine-containing cyclobutane-type photoproducts ($\hat{UT} + \hat{TT}$).

of DNA.[3] His studies indicate that this dimer will only form in denatured regions of DNA. From this model we conclude that the DNA in germinating spores is maintained for a brief period in the hydrogen-bonded B form of DNA before taking on a conformation in which regions of non-hydrogen-bonded DNA are in equilibrium with hydrogen-bonded regions. This model explains the absence of cyclobutane-type dimers in spores but does not explain the appearance of a new thymine photoproduct. A structure for this photoproduct is suggested from the fact that the spore-type photoproduct is formed in dry DNA and dry thymidylic acid (TMP). A dimer involving the 5 and 6 carbons of one thymine and either the 2 carbon and 3 nitrogen or the 4 carbon and 3 nitrogen of an adjacent thymine can form in the A or dry form of DNA. We suggest that a dimer containing one of these azetidine rings is the new photoproduct formed in spores and dry DNA. We have perfected chromatographic techniques for purifying this new photoproduct and will produce sufficient quantities to characterize its structure.

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25.26 SOME STEREOCHEMICAL FEATURES OF THYMIDYLYL DIMERS IN A POLYNUCLEOTIDE

J. W. Longworth

Six stereoisomeric thymine photodimers are possible, two enantiomeric pairs and two meso forms. The isomer formed in DNA is well established as the meso cis-syn isomer. However, there still remain to be established two further stereochemical features. One is the conformation of the cyclobutane ring – whether the ring is planar or twisted. Einstein and Wei[1] have shown that a ring in a related thymine photodimer is planar. The second problem only applies to thymidylyl photodimer and is concerned with the glycosidic conformation. A steric analysis by Haschemeyer and Rich[2] and by Ramachandran[3] has shown that there are only two possible glycosidic conformations in nucleotides. These are syn and anti conformers of the glycosidic linkage, with the anti conformation preferred by pyrimidines. Inspection of space filling molecular model of pTpTp shows that if both thymine rings are in an anti conformation the formation of a cis-syn cyclobutane photodimer is sterically hindered. However, there is no restriction if both thymines are in a syn conformation. It is also to be noted that a twisted cyclobutane conformation in the photodimer would lead to diasteriomerism.

Azetane ring compounds of the kind proposed by Stafford and Donnellan[4] for "spore" photodimers are sterically feasible in an anti glycosidic conformer. A clarification of these academic considerations would be, in part, available from an x-ray crystal structure determination of the cis-syn thymidylyl cyclobutane photodimer.

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25.27 ENERGY TRANSFER IN POLY-L-TYROSINE AT 77°K[1]

J. J. ten Bosch R. O. Rahn
J. W. Longworth

Previous fluorescence measurements of poly-L-tyrosine at 295°K demonstrated a transfer of excited singlet-state energy from the neutral tyrosyl residues to those with an ionized phenolic group.[2] To obtain information on the presence of energy transfer in poly-L-tyrosine at 77°K, we compared the electron spin resonance (ESR) signals of the

excited triplet states with fluorescence and phosphorescence intensities. The measurements were made at 77°K as a function of the percentage of phenolic ionization. *N*-Acetyl-L-tyrosinamide was used as a control instead of tyrosine to avoid complications due to the neutralization of the amino group.

The degree of ionization of the tyrosyl residues in ethylene glycol–dimethyl sulfoxide–water glasses was determined from absorbance measurements at 77°K. An isosbestic point was found at 281 nm (monomer) and 283 nm (polymer). Measurements of the intensities of the fluorescence, phosphorescence, and ESR signals were made with uv irradiation at both 283 nm and a wavelength not absorbed by the neutral species. Monochromators were used in all cases. Fluorescence isoemissive points were found at 327 ± 1 nm for both monomer and polymer with isosbestic excitation.

No energy transfer was found for the monomer. In every case, fluorescence-, phosphorescence-, and ESR-determined triplet-state concentration showed a linear increase of the ionized signal with the percentage ionization for excitation at both wavelengths. The neutral signals showed a corresponding decrease.

In the polymer, energy transfer was demonstrated, because for excitation at 283 nm, fluorescence and ESR signals from neutral residues were completely quenched for values of ionization above 20%. Neutral phosphorescence signals could not be determined separately. All methods showed an enhancement of ionized signals to the value for completely ionized molecules which corresponded to the quenching of the neutral signals. All half-value points were found at 3 ± 1% ionization. Since values for the quenching of singlet and triplet levels were equal and since the triplet is of lower energy than the singlet, energy was transferred within the singlet levels only. When exciting at the wavelength not absorbed by the neutral species, all methods showed the expected linear increase of ionized signal with percentage ionization. Theoretical calculations of the transfer rates are in progress.

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25.28 SPECIFIC GAMMA-RAY EMISSION COEFFICIENT OF ^{137}Cs-^{137m}Ba

M. L. Randolph

The discrepancy between previous theoretical values[1,2] of about 3.1 r cm^2 mc^{-1} hr^{-1} and experimental values[2,3] of about 3.4 of the specific gamma-ray emission coefficient (Γ) for the ionization produced in air by the decay of ^{137}Cs-^{137m}Ba seemed larger than plausible from purely random causes. Hence a careful recalculation of the theoretical value was undertaken.

The value of Γ was calculated from the general expression:

$$\Gamma\left(\frac{\text{r-cm}^2}{\text{mc-hr}}\right) = \frac{4.109 \times 10^{16} e}{W} \sum_i n_i(E_i)\, \mu(E_i)\, E_i\,,$$

where the constant comes from definitions of the hour, curie, and roentgen, e is the elementary charge in coulombs, W the average energy dissipated in air per ion pair formed, $n(E)$ the number of photons of energy E per disintegration (at the point of interest), and $\mu(E)$ the true mass absorption coefficient of the air for photons of energy E. Based on a survey of the literature, the decay scheme given in Fig. 25.28.1 was adopted. For the gamma rays alone the specific emission coefficient was then calculated as 3.21 ± 0.07.

The decay of ^{137}Cs-^{137m}Ba also gives low-energy x-ray lines from the isomeric transition (I.T.). If an absorber is present, as it usually is for gamma-ray measurements both to screen out the electrons and to provide secondary equilibrium, bremsstrahlung is generated as the three electron groups are brought to rest. Calculations

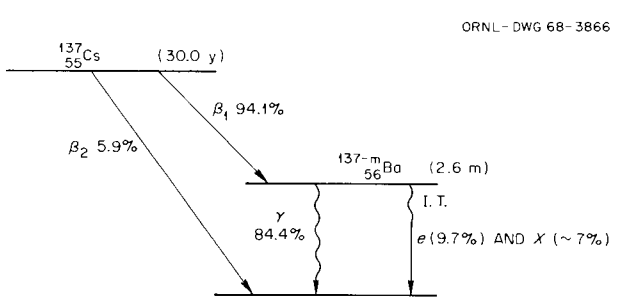

Fig. 25.28.1. Decay Scheme and Branching Ratios of ^{137}Cs-^{137}Ba. All percent values are related to disintegrations of ^{137}Cs.

of these additional photon emissions are incomplete. However, measurements yield directly the specific photon emission coefficient, which arises from the sum of the gamma-ray, x-ray, and bremsstrahlung effects. Thus the overall result of this calculation is to reduce, but probably not eliminate, the discrepancy between the theoretical and experimental values of Γ for ^{137}Cs-^{137m}Ba.

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25.29 IS AGE-DEPENDENT GENETIC DAMAGE IN SEEDS CAUSED BY FREE RADICALS?

A. D. Conger[1] M. L. Randolph

Mutation and chromosomal aberration rates in plants increase markedly with the age of stored seeds from which plants are grown.[2] However, the mechanism is unknown. Our study was designed to test whether such changes are correlated with naturally occurring unpaired electrons as is development of postirradiation damage.[3] Electron spin resonance (ESR) techniques were used to detect unpaired electrons.

Five kinds of dried seeds of ages up to 48 years were contributed by G. E. Nutile (Asgrow Seed Company, Twin Falls, Idaho) and R. A. Nilan (Washington State University). These seeds were carefully dried to the same low moisture content to forestall moisture dependence, such as has been observed in native[4] and irradiated seeds.[3] Electron spin resonance signals in samples of whole or ground seeds were measured with a Varian 4560 ESR spectrometer.

Four classes of ESR signals were found in all seeds tested (Fig. 25.29.1). Three classes probably represent metallic ions and reflect mostly nutritional or species characteristics, rather than seed aging. These three were: (1) strong broad signals at $G = 4.0$; (2) weak 500- to 700-gauss-broad peaks near $G = 2$; and (3) weak 480-gauss-wide patterns of six peaks, which almost certainly represent Mn^{2+} ions. None of these signals were readily power saturated. Their amplitudes (to

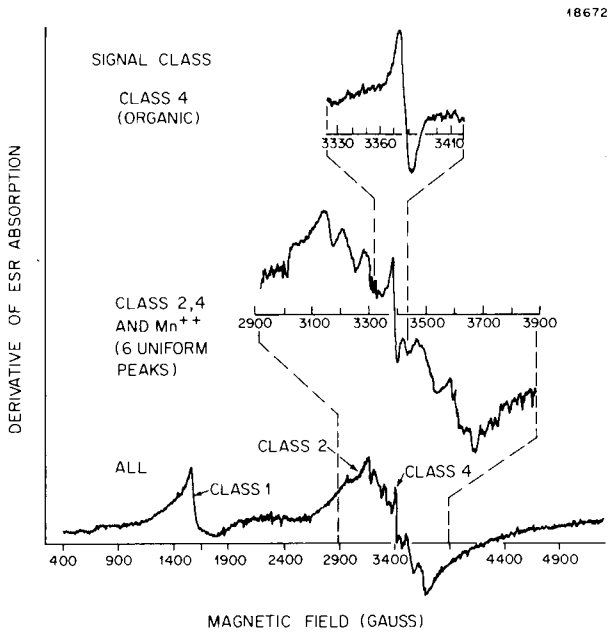

Fig. 25.29.1. ESR Patterns for 1955 Wheat Seed. Classes of signals are described in the text. For instrumental reasons, relative peak heights are not strictly comparable.

±20%) were age-independent within species and differed only twofold between species.

Signals of the fourth class were centered at $G = 2.004$, were 4 to 10 gauss wide (peak to peak), and were clearly power saturable. Since only signals of this class are induced by radiation and are correlated with biological damage, these signals seem most likely to be of biological interest. These signal amplitudes change so slowly with age that the estimated average half-life was ~200 years. Within no species did we observe any fine structure or changes in line shape with age.

Thus demonstrable changes in free radical concentrations or radical species with age of seeds are much less than the concurrent increase in genetic damage. This argues against simple correlation of genetic damage from aging of seeds with unpaired electrons. In this respect damage by aging differs from radiation-induced damage, for which correlations have been found. Finally, in view of our negative results, the notion that aging effects arise from chemical actions of normal metabolites or waste products accumulated slowly during aging appears more likely.[5]

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26. Cooperative Programs

AEC-NCI COCARCINOGENESIS

Biochemistry of Carcinogenesis (26.1, 26.2)

Stanfield Rogers[a]
J. H. Evans[b]
D. W. Parsons
Mary L. Hummel Welch
T. J. Stephens, Jr.
Barbara R. Bussell
Brenda G. Cary
W. F. Fox

Enzymology of Chemical Carcinogenesis (26.3–26.10)

G. David Novelli[a]
Audrey N. Best[a]
Lawrence Rosen[a]
Beryl Ortwerth[a,c]
L. C. Waters[a]
Wen-Kuang Yang[a,d]
Ugo Del Monte[a,d]
Kiki B. Hellman[a]
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Mammalian Chemical Carcinogenesis (26.11–26.13)

A. C. Upton[a]
N. K. Clapp[a]
J. W. Conklin[a]

Chromosomal Effects of Chemicals and Radiation (26.14, 26.15)

E. H. Y. Chu[a]
Patricia A. Brimer[a]
Patricia C. Looby[a]

Effects of Radiation and Chemicals on *Paramecium* (26.16)

R. F. Kimball[a]
E. G. Bailiff[a]

Biophysics of Carcinogenesis (26.17–26.26)

R. B. Setlow[a]
J. R. Einstein[a]
F. C. Hartman[a]
J. W. Longworth[a]
C. H. Wei[a]
I. Lucile Norton[a]

Chemical Mutagenesis in Microorganisms (26.27, 26.28)

F. J. de Serres[a]
H. V. Malling[a]
D. S. Carroll[a]
J. S. Wassom[a]
E. C. C. Gourley[a]

Somatic Cell Genetics (26.29, 26.30)

James D. Regan[a]
W. H. Lee

Inhalation Carcinogenesis (26.31–26.39)

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J. O. Brick
M. G. Hanna, Jr.
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Paul Nettesheim
C. T. Bahner[b]
Alfred Hellman[e]
R. F. Newell[e]
R. W. Presley
O. D. Boyd
J. G. Farrelly[f]
Gertrude H. Brummett
Mary W. Francis
J. L. Grey
Anna S. Hammons
R. J. Holbrook
F. J. Lacina
D. H. Martin
Leona C. Peters
R. C. Satterfield
B. E. Sise
G. G. Smiley
A. K. Szakal
J. R. Webb
Mary Lou Williams
J. E. Whittlesey
L. J. Wapschall
W. L. Dake
A. E. Thomas
C. D. Farmer
Renfro Henderson
J. H. Rather
Helen L. Zang

Biochemical Regulation (26.40–26.45)

F. T. Kenney[a]
J. R. Reel[a,c]
W. D. Wicks[a]
C. B. Hager[a,c]
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[d]Visiting investigator from abroad
[e]Loanee
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[g]USPHS Postdoctoral Fellow

26.1 CONCERNING THE USE OF VIRUSES TO TRANSMIT GENETIC INFORMATION

Stanfield Rogers

The Shope papilloma virus induces a virus-specific arginase in rabbit squamous cell epithelium. A lowered blood arginine is a systemic effect associated with such infection, which induces papillomas in the skin. When the virus is injected intravenously through slits so as not to induce papillomas, the rabbits still develop low blood arginine levels, indicating that there is a systemic infection with the virus. In studies recently reported[1] it was found that the blood arginine level could be used as a determinant in man as to whether or not he had worked with this virus. About half the individuals having had contact with the virus could be separated out blind from normals through changes in their blood arginine levels. Such use of the induction of arginase as a marker for the virus, indirectly through their blood arginine levels, is now being studied in mice, rats, dogs, and, in conjunction with J. Melnick's group in Houston, baboons and monkeys. Results are complete only in mice and rats, both of which develop low blood arginines after infection with the virus, like domestic rabbits, wild cottontail rabbits, and people. These findings plus other inferential data derived from physical and chemical studies of rabbit liver and papilloma arginase, including sedimentation velocity, partial specific volume, molecular weight, peptide patterns, movement on carboxymethyl cellulose columns, strongly suggest that the enzyme is derived from virus synthetic information rather than from that of the infected mammals listed. A more direct approach to this problem is provided by a mutant line of the Shope virus, which unlike wild type is recoverable in the domestic rabbit. It was determined to find whether the mutation in virus information associated with its recoverability resulted in a structural change in the enzyme.

The free arginine pool of the tumors induced with the recoverable line is increased considerably over that found in wild-type-induced tumors. This is also associated with a reduction in the amount of ornithine formed in relation to arginine taken up, indicating that the recoverable-line-induced papillomas have less arginase activity than wild-type tumors of the domestic rabbit. Upon purification of this mutant-virus-induced arginase, using the standard system used for the enzyme induced with wild-type virus, it was found that the enzyme activity came off carboxymethyl cellulose much later than wild type and required about twice the molarity of the eluting buffer. Studies at sedimentation equilibrium revealed two components, a small amount having a molecular weight of 43,000 and a large amount having a molecular weight of 400,000. The specific activity of this mixture of the two components when free of manganese had only about 15% of that expected from wild-type enzyme as judged from protein concentration. The mixture was then put through Sephadex G-200 to separate the two components. The 400,000-molecular-weight material came off just after the void volume and had no activity until activated with manganese, a step never required for wild-type enzyme. The 43,000-molecular-weight enzyme appears to be the monomer broken off the larger polymer and acts in relation to manganese like wild-type arginase. It was reported last spring by S. Sorof at the ACR meeting that the monomer of liver arginase (molecular weight under 20,000) does not require manganese activation, while polymers thereof do. Studies are under way to find whether a similar polymer of papilloma arginase may exist in wild-type virus-induced tumors or whether, as our previous studies indicate, only the 43,000-molecular-weight enzyme exists in these. In any event the structures of the enzymes induced by the two virus lines are sufficiently different to be widely separated on carboxymethyl cellulose. This difference structurally must be related to differences in the original monomers modifying either the formation or stability of the polymer. In optical rotatory dispersion, the mutant enzyme has a somewhat less, probably insignificant, helical structure than the wild type as judged from the *M'*.

Rabbit liver infected with wild-type virus was examined to find if the virus-induced enzyme could be isolated, if so accounting for the systemic effect of the virus on the blood argine levels. An arginase moving on carboxymethyl cellulose like the virus-induced enzyme and separated widely from liver arginase was found. This purified enzyme has the optical rotatory dispersion characteristics of virus-induced enzyme.

Though it is conceivable, but without precedent, that the mutant virus induces the modified enzyme

from rabbit information, these results greatly increase the probability that the enzyme is derived from virus information and that the mutation of the virus is directly associated with the change in structure of the enzyme.

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26.2 THE ISOLATION OF POLY-LYSINE INDUCED BY POLY-ADENINE-MODIFIED TOBACCO MOSAIC VIRUS RNA IN TOBACCO PLANTS

Stanfield Rogers Peter Pfuderer

The observation that the Shope papilloma virus specifically lowers the blood arginine levels in wild and domestic rabbits and in man suggests that virus information may be used in the control of genetic deficiency diseases and perhaps other diseases, including neoplasia. Theoretically one may use information innately part of the virus, that specifically transduced from other genomes, or perhaps that synthesized in the laboratory and attached to the virus DNA or RNA, using the virus as a vector. As a test of this latter hypothesis, it was decided to add nucleotide sequences to a virus RNA or DNA and find whether any specific effects of such information could be detected in infected cells. The tobacco mosaic virus seemed the agent of choice, as the RNA itself is infective and can be purified readily in relatively large quantities.

The RNA of the tobacco mosaic virus was purified using the standard methods of Stanley and associates. Using the methods of Singer, the RNA was treated with polynucleotide phosphorylase in the presence of either uridine diphosphate or adenosine diphosphate, with the modification of added pyrophosphate to push the reaction toward synthesis of polynucleotides. This enzyme requires an RNA primer which it incorporates into the synthesized polymer. When ^{14}C UDP was used and the RNA washed in the centrifuge after treatment, all the radioactivity was found associated with the RNA.

Both Xanthi and Turkish tobacco plants were inoculated using standard methods. The Xanthi variety develops only local lesions, while the Turkish develops systemic disease and synthesizes larger amounts of virus. Only the bottom two to four leaves of the Turkish were inoculated. After gross disease was evident, the leaves were homogenized and the protein precipitated with TCA and the soluble basic proteins precipitated with the addition of tungstic acid following the methods of Ochoa. The basic proteins were then analyzed for the presence of poly-lysine. Control trypsin digestions on poly-lysine showed that the enzyme reaction reached a plateau after roughly 20 min, producing maximal amounts of tri and tetra lysine, with smaller amounts of the larger and smaller fragments. Tryptic digestions were then performed on the basic proteins derived from poly-A-RNA infected and control normal TMV RNA infected plants. The products were characterized by one- and two-dimensional chromatography. Lysine containing peptides which co-chromatograph with mono, di, tri, tetra, and penta lysine have been found in the material from the poly-A-RNA infected plants. The control material yielded lysine containing peptides only in the monomer position and a trace amount which moved like trilysine. As some other amino acids were encountered in these peaks another step was added. Using the medium column of the Beckman amino acid analyzer, it was found that lysine and its polymers were eluted at the same time and could thus be separated from peptide contaminants. The tungstic acid precipitated material was dialyzed, the dialysate concentrated and put on this column, the "lysine" peak collected, the acetate buffer then converted to acetic acid and sodium chloride, the acetic acid evaporated off, and the sodium chloride removed using ion exchange retardation resin (Bio-Rad AG11A8). The eluate was concentrated and put through the carboxymethyl cellulose column as before. Background amino acids were reduced using special distilled water, fired glassware, etc. Tetra and pentameric lysines, judging from their position of elution from CM using a linear sodium chloride gradient, were found. Upon hydrolysis, 60 molecules of lysine were found for each arginine recovered in the tetrameric material and 32 for each arginine in the pentameric material. Arginine was the only contaminant detected.

26.3 ETHIONINE CARCINOGENESIS

Beryl Ortwerth G. David Novelli

The metabolism of ethionine has been studied for many years in an effort to explain its hepatocarcinogenic activity. It has been reported that the ethyl group of ethionine is transferred to the nucleic acids of rat liver in vivo. This activity has been studied in our laboratory and appears to be restricted to tRNA with little or no incorporation into DNA or rRNA.

The major reaction of ethionine in vivo is its conversion to *S*-adenosylethionine (SAE). This compound has been suggested as the active intermediate in the ethylation of tRNA. Our results indicate that SAE is not the active intermediate because:

1. the distribution of ethyl groups among the tRNA's is different from the normal distribution of methyl groups which is known to proceed via *S*-adenosylmethionine,
2. the labeling of tRNA by ethionine is maximum after 3 hr and is not present at 24 hr, even though the pool of *S*-adenosylethionine is unchanged,
3. the injection of methionine, which is known to prevent *S*-adenosylethionine formation, has no effect on the ethylation of tRNA.

The ethylation is not a random substitution of ethyl for methyl groups on the tRNA and apparently does not react with all species of tRNA. We have also shown that tRNA synthesis is required before ethylation can proceed presumably to provide a suitable undermethylated substrate. tRNA was isolated from normal rat liver, from livers of rats fed an ethionine-containing diet for five months, and from ethionine-induced hepatomas. These tRNA's were compared by cochromatography of their amino acylated derivatives on the Freon reversed-phase system. Of the amino acids tested, no change was seen with methionine, aspartic acid, glycine, threonine, tyrosine, and proline. tRNA's from ethionine-fed rats had small new peaks with phenylalanine, leucine, and lysine and larger quantitative shifts with alanine, histidine, tryptophan, and arginine. The profiles of the tRNA's from hepatoma tissue are currently being studied to see if any of the changes above have become permanent.

26.4 ETHYLATION OF HEPATIC tRNA BY ETHIONINE IN VIVO

Lawrence Rosen Shigemi Iiyama
G. David Novelli

Following the injection of ^{14}C-ethionine (ethyl labeled), the tRNA fraction of the liver is labeled, whereas other hepatic nucleic acids are insignificantly labeled, if at all. In contrast to other alkylating agents,[1] ethionine yields many different ethylpurines and pyrimidines.[2] Some evidence has accumulated that the ribose moieties are also ethylated, presumably at the 2′-*O*-position. The different labeled compounds have been separated by ion exchange and paper chromatography. Various ethylpurines and pyrimidines have been synthesized and characterized to aid in the identification of the different ^{14}C-compounds.

Acid hydrolysis of the ^{14}C-ethyl-RNA indicates that 45 to 50% and 50 to 55% of the radioactivity is in the purine and pyrimidine fractions respectively. The ethylated purines may be separated into six different components, and 7-ethylguanine has been positively identified. It accounts for 8 to 12% of the total radioactivity. 3-Ethyladenine has been tentatively identified and accounts for approximately 10% of the label. On the basis of ion exchange chromatography 1-ethyl-, N^6-ethyl-, and N^6,N^6-diethyladenine have been excluded as components of the ethylpurine fraction.

The labeled pyrimidine nucleotides have been separated into two fractions, the ethylated cytidylic (EtCp) and ethylated uridylic (EtUp) acids, each of which has two or more components. Each of these fractions accounts for approximately 16% of the original radioactivity. More than 70% of the EtCp fraction can be enzymatically dephosphorylated to yield three different nucleosides. Following hydrolysis of the EtCp fraction with 11 *N* PCA (sealed tube, 100°) 80 to 85% of the radioactivity may be distilled in vacuo. This treatment with PCA should hydrolyze *N*-ethyl,[3] *O*-ethyl,[3] and 2′-*O*-ethyl groups[4] to ethanol, which would be distillable in vacuo. On the basis of this experiment it appears that only 15 to 20% of the radioactivity could be due to C-ethylation, such a possibility being 5-ethylcytidylic acid, an analog of the naturally occurring 5-methylcytidylic acid.

Following hydrolysis of the EtUp fraction with PCA, 80 to 85% of the radioactivity could also be

distilled in vacuo, again indicating the presence of *N*-ethyl, *O*-ethyl, and/or 2′-*O*-ethyl groups. In this case only 20 to 25% of the EtUp fraction could be enzymically dephosphorylated. Since it is possible that a bulky 2′-*O*-ethyl group would prevent the enzymic removal of an adjacent *cis*-3′-phosphate group, this may indicate that the ETUp fraction contains a much greater proportion of 2′-*O*-ethyl groups than the EtCp fraction. A possible component of the EtUp fraction due to C-ethylation could be 5′-ethyluridylic acid, an analog of the naturally occurring ribothymidylic acid.

A radioactive alkali-stable dinucleotide fraction was isolated from ethyl-^{14}C-tRNA. Insignificant, if any, labeling of purines was found in this fraction, the radioactivity essentially being found in only the pyrimidine nucleotides of this fraction. Stability to alkaline hydrolysis is conferred by a 2′-*O*-alkyl moiety such as the natural 2′-*O*-methyl group, but the possibility of 2′-*O*-ethylation also occurs. Whether alkaline stability is due to 2′-*O*-methyl or 2′-*O*-ethyl groups is irrevelant to the conclusion that may be drawn from this experiment. This is that ethylated purines are not, but ethylated pyrimidines are, involved in a glycosyl bond to a 2′-*O*-alkylribose moiety and/or are involved in a 5′ → 3′-phosphodiester link to such a moiety. This indicates a degree of specificity for ethylation sites in tRNA despite the multiplicity of ethyl compounds formed.

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26.5 BIOCHEMICAL STUDIES IN RAT LIVER DURING ETHIONINE CARCINOGENESIS

Ugo Del Monte G. David Novelli

The livers of rats that have been fed an ethionine-containing diet for five months develop a nodular hyperplasia that finally results in neoplasia. From a general point of view, it is important to point out that, contrary to other well-known chemically induced hepatic tumors, ethionine-induced hepatomas arise almost exclusively from a single type of cell, that is, the parenchymal cells, thus providing an ideal model for studying the biochemistry of carcinogenesis in liver. Among other biochemical processes influenced by ethionine, recent attention has been focused upon the possible alteration of liver transfer RNA by alkylation. The present study is designed to determine alteration in tRNA and aminoacyl RNA synthetases during ethionine carcinogenesis.

The aminoacyl-tRNA synthetases were prepared from a 100,000 × *g* supernatant fraction by DEAE-cellulose-column chromatography followed by gel filtration. The tRNA fraction was eluted from the same DEAE column using a higher salt concentration and was further purified by a phenol method. On the basis of the wet weight of the tissue, in nodules and in hepatomas there is a decrease in the amount of tRNA, possibly due to an increased tissue hydration. The tRNA's from liver, nodules, and hepatomas were aminoacylated in vitro under optimum conditions using synthetases from the same source. The leucine acceptor activity of tRNA is decreased in both nodules and tumors. Furthermore, in either tissue the relative proportions between isoaccepting $tRNA^{Leu}$ are different from the normal, as shown by cochromatography on reversed-phase Freon columns of tRNA's from normal and pathological tissue (aminoacylated respectively with ^{3}H- and ^{14}C-labeled leucine). Finally, results of cross reactions and of reactions carried out in the presence of different amounts of magnesium indicate that following ethionine feeding some changes occur also at the synthetase level.

The methionine acceptor activity of tRNA's from liver, nodules, and hepatomas is about the same. Using the synthetase from normal liver it has been shown that the V_{max} of methionyl-tRNA formation is practically normal in the case of the tRNA from nodules, while it is consistently decreased in the case of tRNA from hepatomas. Possible reasons for this result are currently being investigated. A study on tRNA methylases has been undertaken in this connection to see whether the change observed in V_{max} may be related to any difference in the extent and/or pattern of methylation of tumor tRNA.

26.6 tRNA'S IN RAT SPLEENS DURING IMMUNE RESPONSE

Wen-Kuang Yang G. David Novelli
C. J. Wust

Recently a possible control mechanism of immunoglobulin biosynthesis at the step of amino acid activation was discussed.[1] This postulate was further developed into the so-called "permissive translation mechanism" for antibody formation.[2] Experimental proof or disproof of this postulate is not available due to difficulties in studying amino acid activation in the antibody-forming tissues. Attempts were initiated for the preparation of tRNA's and aminoacyl tRNA synthetases from rat spleens. It was found that the methods devised for the mouse plasma cell tumor tissue could be adapted for the spleen. The amount of tRNA in the supernatant fraction was found to be very low, but a large portion of tRNA was found to be associated with the microsomal fraction of spleen. Chromatographic patterns on the reversed-phase Freon column were found to be similar for seryl-tRNA's isolated from the supernatant and the microsomal fractions.

Groups of rats were immunized with sheep erythrocytes and sacrificed 4 days, 7 days, and 14 days after primary inoculation and 7 days after a secondary challenge. Spleens were isolated and used for preparing tRNA and aminoacyl-tRNA synthetases. Comparative studies of seryl and leucyl tRNA's by cochromatography on the reversed-phase Freon column showed that there were only quantitative changes in the isoaccepting species of these tRNA's in the spleen after immunization without any qualitative modifications. The quantitative changes were minor and consistently found in the tRNA's prepared from the supernatant fraction, microsomes, or whole tissue. Preliminary results from aminoacylations of tRNA mixtures with serine, leucine, methionine, and arginine in the presence or absence of the other 19 amino acids indicate that it is unlikely that changes in these tRNA's take place following immunization.

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26.7 tRNA'S OF L-M CELLS IN IN VITRO CULTURE AND IN IN VIVO TUMOR

Wen-Kuang Yang D. H. Martin
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G. David Novelli

Mouse L-M cells can be cultured in large scale in vitro and also can initiate tumor formation in vivo when inoculated into C_3H mice.[1] It became apparent to us that this system could be used for studying amino acid activation in the same tissue under different environmental conditions. Initial results showed that the methods devised for the plasma cell tumor tissue[2] could be adapted for preparing tRNA and aminoacyl-tRNA synthetases both from the cultured L-M cells and the tumor L-M cells. The content of tRNA and the enzyme from the two tissues were similar and cross-reacted quantitatively for amino acid activation. To examine closely if the aminoacyl-tRNA synthetases from L-M cells and L-M tumor may be different, tRNA's from both tissues were chromatographed on the reversed-phase Freon column, and the accepting activities assayed by the two enzyme preparations were compared. Identical patterns were obtained for most amino acids except arginine and tyrosine, which showed possibly minor differences.

Comparisons of aminoacyl-tRNA's from the L-M cells and L-M tumors of male and female mice are in progress. Initial results showed that this tissue has its unique patterns of isoaccepting tRNA's. Leucyl, lysyl, and arginyl tRNA's were similar in vitro and in vivo. A component of seryl-tRNA's (seryl-$tRNA^{IV}$) is present in the in vitro L-M cells but is undetectable in the in vivo L-M tumors. A prominent peak of tyrosyl tRNA's (tyrosyl $tRNA^{I}$) was found in the L-M tumor but was absent in the L-M cells. These results led to a tentative conclusion that some modifications of tRNA may occur in a tissue when environmental conditions change.

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26.8 ISOLATION OF RIBOSOMAL RNA FROM RAT SPLEEN

C. J. Wust D. G. Ridner

The typical absorbancy profile of ribosomal RNA of rat spleen after centrifugation through density gradients of glycerin reveals a small amount of material at 4S and at 5 to 12S and large amounts at 18S and 28S. In addition there are two peaks at 40S and 41S, the former being quantitatively similar to 18S. All the peaks are susceptible to digestion with ribonuclease but not DNase.

These preparations have little amino acid acceptor activity, and they do not stimulate amino acid incorporation onto ribosomes in a cell-free system. No differences could be found in the profiles or yields between ribosomes from normal and immunized animals, although the amount of ^{32}P incorporated by ribosomes from immunized animals is three to four times higher at two days after injection of antigen. The incorporation of ^{32}P-orthophosphate into the RNA indicated further that the 40S and 51S are not labeled during short periods of time. This suggests that they are long-lived species and are not precursors of the 18S and 28S. The RNA can be subdivided into at least two main groups based on solubility in 1 *M* NaCl. RNA soluble in the salt was found to be predominantly 4S-tRNA that readily accepts amino acid. The reason that the tRNA could not be demonstrated before salt extraction is not known at present. The fraction that is precipitated by 1 *M* NaCl was shown to contain ~8S, 18S, 28S, 32S, 40S, and 51S as individual peaks. The 32S labels with ^{32}P at a rate consistent with the concept that it is a precursor to the 28S and 18S.

Ribosomal RNA, that is, 18S, 28S, and 40S, has been found in the supernatant of microsomal preparations treated with deoxycholate. This finding indicates that deoxycholate not only solubilizes the endoplasmic reticulum but some ribosomes as well. This may be a selective solubilization since we have reported previously[1] that rapidly labeled RNA is solubilized with this bile salt detergent. Ribosomal RNA may be isolated in the supernatant fluid of ribosomal preparations made when 10^{-3} *M* or 10^{-5} *M* Mg^{2+} is used in the homogenizing medium, being in greater quantity in the latter instance.

We conclude that there is a diversity of RNA species in cytoplasmic ribosomes of rat spleen. Several of these species have not been reported in other mammalian systems.

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26.9 RIBOSOMES OF RAT SPLEEN

C. J. Wust D. G. Ridner

We have reported previously[1] that two classes of ribosomes can be isolated from the spleens of rats. One class is in the supernatant fluid of the homogenized spleen, and the other is derived from treatment of the 10,000 × *g* pellet (unbroken cells, nuclei, and mitochondria) with Triton X-100. It became apparent that although the optical density profiles of these ribosomes after zonal centrifugation were consistently reproducible, there were instances in which some variation was found in the relative amounts of the different sizes of ribosomes, especially in the 65S, 81S, 105S, and 120S regions. We had further observed from previous work[2] that the 65S and 105S peaks were not labeled to any extent over a 2-hr pulsing period with ^{32}P. Other observations with actinomycin suggested that the 65S was a degradation subunit because it was the prominent remaining peak after treatment with the drug. We had also reported[3] that when anti-L chain labeled with ^{125}I combined with polysomes derived from spleens of normal or immunized rats, there was a lack of coincidence between the optical density profile and radiospecific activity. These various findings suggested that much of a polysomal optical density profile might be an expression of some anomalous condition of isolated particles. Our effort has been to evaluate the influence of our extraction procedures on the state of native ribosomes.

Ribosomes from spleens of rats were isolated in homogenizing media containing Mg^{2+} at 10^{-2}, 10^{-3}, or 10^{-5} *M*. Compared with the usual profile found with 10^{-2} *M*, 81S was the predominant peak in the profile at 10^{-3} *M*, with lesser amounts of 120S, 105S, and 65S. If the Mg^{2+} concentration was increased to 5×10^{-3} *M* before zonal centrifugation, the 105S and 120S were increased with a

concurrent decrease in the 65S and 81S. When the homogenizing medium contained 10^{-5} *M* Mg^{2+}, the predominant peak was the 65S with some 81S and 50S. Treatment with EDTA, 10^{-2} *M*, of the ribosomes isolated in 10^{-2} *M* Mg^{2+} resulted in a breakdown to 50S and 30S subunits in a ratio greater than 2:1. Such treatment of ribosomes isolated with 10^{-5} *M* Mg^{2+} gave the same subunits in a ratio of about 1:1.

Tentatively, we conclude that much of the profile is magnesium dependent; the 105S is a magnesium dimer of the 65S, and the 120S is a magnesium dimer of the 81S; subunits of spleen ribosomes are 50S and 30S; a 65S and 30S combine to give an 81S; the 65S is a stable subunit and might be composed of two 50S subunits. These interpretations reflect on all optical density profiles found with ribosomes isolated from other cell populations.

Although the integrity of the ribosomes is largely magnesium dependent, the amount of polysomes greater than 120S also appears to depend upon the presence of RNA. Almost no polysomes are observed when the homogenizing medium contains 10^{-3} *M* Mg^{2+}. The supernatant fluid contains ribosomal RNA as well as tRNA, and the supernatant fluid of the preparation made with 10^{-5} *M* Mg^{2+} contains significantly more RNA. The addition of Mg^{2+} plus this RNA reconstitutes some of the polysomal optical density in the ribosomes isolated with 10^{-3} *M* Mg^{2+} but not those isolated in 10^{-5} *M*, although Mg^{2+} by itself restores a good quantity of 154S and 175S.

We have found that the yield of ribosomes per gram of spleen is dependent on the Mg^{2+} concentration in the homogenizing medium; that is, the higher the concentration, the greater the yield and the higher the relative amount of 105S and 120S. However, treatment of the 10,000 × *g* pellet with Triton X-100 gives ribosomes inversely dependent upon Mg^{2+}, so that the total yield of both classes is independent of Mg^{2+}.

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26.10 STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF tRNA PYROPHOSPHORYLASE

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We have continued the investigation of the purification of the enzyme(s) that incorporates AMP and CMP from ATP and CTP into the terminal position of tRNA:

$$\text{tRNA ---- + ATP + 2CTP} \rightleftharpoons \text{tRNA ---- pCpCpC + 3PP .}$$

The substrate tRNA for the initial assays of the enzyme was prepared from a partially purified tRNA fraction from *Escherichia coli* W by periodate oxidation followed by amine cleavage and subsequent treatment with alkaline phosphatase. The assay measures AMP incorporation into this oxidized tRNA preparation. A procedure consisting of treating the cell-free extract of *E. coli* B with streptomycin sulfate, precipitating the active protein with $(NH_4)_2SO_4$ between 0.3 and 0.5 saturation, followed by successive chromatography on DEAE-cellulose and carboxymethyl cellulose (or phosphocellulose) will give a preparation of perhaps 50% purity. Electrophoresis of several purified preparations in polyacrylamide gel at pH 8.5 showed one major protein band and 4 to 6 minor bands. The problem of the removal of the remaining contaminating proteins has not been solved completely. An enzyme preparation that had been purified through the DEAE-cellulose chromatography step was tested on a small scale (10 to 15 mg protein) by further chromatography on DEAE–Sephadex A-50, carboxymethyl–Sephadex C-50, G-100, and hydroxylapatite. The efficiency of each chromatographic procedure was evaluated by electrophoresis studies at pH 8.5 on cellulose acetate membranes. A comparison of the distribution and number of the protein bands indicated that the most promising procedures were chromatography on DEAE–Sephadex A-50 at pH 8.4 and on hydroxylapatite at pH 6.5. At the present time we are attempting to obtain a more than 50% pure protein by successive chromatography on DEAE–Sephadex (pH 8.4) and hydroxylapatite (pH 6.8).

We have been trying to improve the purification in the earlier stages. It is possible to outline tentatively the following steps that can be followed for the large-scale purification of this enzyme:

1. rupture of the cells by the Gaulin press,

2. precipitation of nucleic acids with streptomycin sulfate,
3. precipitation of protein between 0.3 and 0.5 $(NH_4)_2SO_4$ saturation,
4. further purification by treatment with $MnCl_2$ and/or $CdCl_2$,
5. DEAE-cellulose chromatography, pH 6.8,
6. batch treatment with carboxylmethyl cellulose or phosphocellulose, pH 6.5,
7. second $(NH_4)_2SO_4$ precipitation,
8. DEAE–Sephadex A-50 chromatography, pH 8.4,
9. hydroxylapatite chromatography, pH 6.8.

Step 6 might be necessary only when large-scale preparations are made. Although the pyrophosphorylase is stable at pH 6.5, some other proteins precipitate at this pH, causing complications and erratic results during chromatography. We are planning to test this outlined procedure in the near future using fresh (unfrozen) *E. coli* B cells.

26.11 PULMONARY AND HEPATIC ONCOGENESIS AFTER DIMETHYLNITROSAMINE (DMN) TREATMENT OF MALE RF MICE

N. K. Clapp A. W. Craig[1]
R. E. Toya, Sr.

Dimethylnitrosamine (DMN) is a potent carcinogen to the liver in the rat, affecting secondarily in frequency the kidney and lung; however, relatively little work has been reported in inbred mouse strains. This study was undertaken to define the carcinogenicity of DMN in mice by determining the sites, cell types, and incidences of tumors, as well as the time when tumors could first be diagnosed.

A total of 125 male RF mice 8 to 10 weeks of age were treated with DMN in the drinking water continually for their lifetimes. The mice consumed 0.94 mg of DMN per kilogram body weight per day, with a mean cumulative dose of 297 mg/kg. Randomly selected mice were sacrificed at monthly intervals after treatment began, while the remaining mice were necropsied at death or sacrificed in a moribund state. The first death occurred at 8 months of age, and the last mouse died at 18 months. The mean age at death in treated mice was 13.0 months, as compared with 20 months for nontreated controls, a life shortening of 247 days or 37%. The sigmoid-shaped mortality curve resembled that of control mice but was much steeper in slope. The cumulative tumor incidence in organs of dying mice was as follows: liver, 97%; lung, 99%; and stomach, 2% (Table 26.11.1), as compared with respective control values of 3% (liver), 39% (lung), and 2% (stomach). The lung tumors resembled the spontaneous pulmonary tumors of control mice, whereas the liver tumors were predominantly hemangiosarcomas (2% hepatomas). The latter contrasted with the high incidence of hepatomas in RF mice induced by diethylnitrosamine (DEN) treatment.[2] Hemangiosarcomas of the liver were diagnosed only slightly earlier (after 7 to 8 months of treatment) in sacrificed mice than in those that died, suggesting that DMN-induced liver tumors developed rapidly and were a contributing factor in the death of the animals. These erythrocyte-rich tumors frequently ruptured terminally, producing a hemorrhagic ascites. Sacrificed mice had lung tumors as early as four months after treatment was begun and had a high incidence before liver tumors were diagnosed. Two mice had myeloid leukemia (2%), and seven had reticulum-cell sarcoma (6%), incidences comparable with those of controls of similar age distribution. The incidence of stomach tumors in DMN-treated mice was similar to the control level, in contrast to the high incidence of tumors in the forestomach and esophagus of DEN-treated mice.[2] No leukemogenic effect was observed after either DMN or DEN treatment.

Table 26.11.1. Comparative Incidence of Tumors Induced by Dimethylnitrosamine (DMN), as Compared with Diethylnitrosamine (DEN), in Male RF Mice

	DMN	DEN[a]	Controls
No. of mice	93	42	162
Mean dose, mg kg^{-1} day^{-1}	1	6	
Survival, months	13	9	20
Tumor incidence, %			
Lung	99	74	39
Liver	97[b]	90[c]	3
Stomach	2	100	2
Esophagus	0	18	0

[a]From ref. 2.
[b]Predominantly hemangiosarcomas.
[c]Predominantly hepatomas.

Although similarities in oncogenic effects of these closely related compounds are, therefore, evident from our results, there are also intriguing differences in the target organs and in tumor types that remain to be explained. The extent to which these differences may reflect organ variations in concentration or metabolism of the compounds deserves to be explored.

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26.12 DIETHYLNITROSAMINE CARCINOGENESIS IN BALB/c MICE

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J. A. Otten

It has been previously demonstrated that mice immunized with Rauscher leukemia virus (RLV) vaccine are protected against challenge with RLV-induced myxofibrosarcoma cells.[1] Since leukemia viruses are common contaminants of various murine tumors, the possibility that immunization with RLV vaccine might protect to some extent against tumor induction by diethylnitrosamine (DEN) was tested. Sixty 17-week-old male BALB/c mice were immunized with either RLV vaccine or control vaccine and then treated with DEN continually in the drinking water for 21 weeks. Since, as shown in a companion report, no difference in tumor incidence was observed between the RLV and control groups, the mice were pooled to evaluate DEN tumorigenicity as compared with that previously observed in RF mice subjected to similar DEN treatment.[2]

Survivors of the treatment period consumed an average cumulative dose of 871 mg of DEN per kilogram body weight (6 mg kg^{-1} day^{-1}). At 39.5 weeks of age, 37% of the mice were dead, and most of the survivors appeared to be terminally ill. To avoid loss of histological information on tumor incidence, the remaining mice were sacrificed. The cumulative mortality curve prior to the time of sacrifice was similar to that obtained in DEN-treated male mice of the RF strain,[2] whereas, by contrast, nontreated BALB/c males have a cumulative mortality of only 2% at 40 weeks, and their curve does not reach 40% until they are 80 weeks of age. Mice dying after 17 weeks of treatment had a high incidence of tumors, that is, 21% (13/60) in the liver, 52% (32/60) in the stomach, 3% (2/60) in the lung, and 6% (4/60) in the esophagus. Nontreated male BALB/c mice had <1% incidence of spontaneous tumors in each of these organs through 80 weeks of age. The liver tumors were hemangiosarcomas (one hepatoma was observed); the pulmonary tumors were adenomas and papillary adenomas; the forestomach and esophageal tumors were predominantly squamous cell carcinomas, with an occasional papilloma. Only one mouse died with leukemia (2%), a reticulum-cell sarcoma, which gives in BALB/c mice, as in the RF male, no suggestion of a leukemogenic action of DEN.

The sensitivity of the forestomach of the BALB/c mouse to induction of squamous cell carcinoma by DEN is similar to that of the RF mouse[2] and different from that of the DBA mouse (no stomach tumors), as observed by Schmahl *et al.*,[3] and that of the C3H and ICR mouse strains (papillomas), as observed by Takayama and Oota.[4] The principal type of liver tumors induced by DEN in RF mice and other laboratory animals is the hepatoma. The BALB/c mouse has shown little susceptibility to lung tumor induction, in contrast with the RF mouse, which is highly susceptible to pulmonary tumorigenesis by DEN. These strain differences point to factors, as yet undetermined, that influence the incidence, site, and types of tumors within the target organs of mice following DEN treatment.

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26.13 ALTERED ESTERASE ZYMOGRAMS ASSOCIATED WITH LEUKEMIA VIRUS INFECTION

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It was previously observed that infection of an established cell culture (JLS V6) with Rauscher leukemia virus (RLV) results in a morphological transformation and concomitant increase in tumorigenicity, mucopolysaccharide synthesis, and phagocytosis. To study the underlying metabolic alterations in the infected, transformed cultures (JLS V5), we undertook to characterize several enzyme patterns in such cell cultures and more recently in RLV-infected leukemic mouse spleen tissue.

The first enzymes examined were the nonspecific esterases. For this purpose, infected JLS V5 and control JLS V6 cells were cultured on standard 75×35 mm glass slides. Before the cell sheet became confluent (24 to 48 hr after subculturing), nonspecific esterase activity was determined histochemically with α-naphthyl butyrate as substrate and Fast Blue RR salt as the diazo coupling agent. The slides were then examined microscopically for insoluble Fast Blue RR–α-naphthyl complex, as an indication of esterase activity. In addition, washed cell pellets of control (JLS V6) and infected (JLS V5) cultures were homogenized with tissue grinders, the cell-free extracts were subjected to electrophoresis on acrylamide gel, and the esterase activity in the gel determined with α-naphthyl butyrate as the substrate. Zymograms of extracts from normal BALB/c spleen and RLV-infected leukemic spleens were also prepared in like manner. All resultant esterase zymograms were quantified densitometrically.

Examination of the slide preparations revealed a marked increase in total esterase activity in the infected JLS V5 cells. Furthermore, in three separate experiments the zymograms from the extracts of infected cells showed a 15- to 30-fold increase in total esterase activity per milligram of protein, which was attributable to the appearance of at least six additional electrophoretically distinguishable esterases. Analysis of the zymograms of infected leukemic spleen extracts likewise revealed increased esterase activity and additional esterase bands as compared with normal spleen extracts.

The significance of the alterations in esterase activity associated with RLV infection cannot be fully assessed at this time. Both in vivo and in vitro studies are presently under way to determine if the "new" esterases are associated with virus production per se or represent the selective growth advantage of the virus-infected cells within a mixed cell population. It is also important to determine if the "new" esterases are coded for by the virus genome or merely represent de-repressed cell esterases and whether the appearance of such esterases precedes leukemia in virus-infected mice.

26.14 MAMMALIAN CELL GENETICS. I. SELECTION AND CHARACTERIZATION OF MUTATIONS

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Patricia A. Brimer

The long generation time for man and the difficulty of experimentation have restricted the progress of human genetics. With the use of cell culture techniques, the human and other mammalian somatic cells in culture have been used instead of intact organisms for genetic studies. Essentially there are two sources of genetic markers: (1) cells from organism with a known genetic mutation and (2) mutants selected in established cell lines. The limitations of the former approach are, among others, the rarity of affected individuals, the lack of expression of the phenotype at the cellular level, and technical difficulties of handling diploid cell strains. Most of the established cell lines are heteroploids, which are undesirable for theoretical and practical reasons.

We have chosen an established cell line (V79-129D1) of Chinese hamster (*Cricetulus griseus*, $2n = 22$) as a model system for somatic cell genetics because of its relatively simple karyotype at a near diploid level, active growth, high plating efficiency, and adaptibility to growth in a defined medium. Lacking information in the formal genetics of this species, we initially undertook to isolate naturally occurring genetic variants in cell cultures by various selective techniques. Mutants recovered through the use of two such techniques will be described here.

Results. – *Glutamine Auxotrophy (gln⁻).* – The first selective technique makes use of the differential growth of prototrophs and auxotrophs during a state of thymine deficiency created by aminopterin.[2] Those mutant cells that are incapable of growth because of an amino acid deficiency survive, whereas the normal cells are killed by growth under thymine-deficient conditions. The method should be applicable in the selection of auxotrophs for any amino acids that are nonessential for most mammalian cells. We, however, have not been successful in several attempts to obtain such mutants with the exception of auxotrophs for L-glutamine and possibly those for L-proline. The surviving fraction after six days in the selective medium lacking L-glutamine was 3×10^{-4}. A number of clones (36 out of 48 isolated in one experiment and 12 out of 20 in another) have been recovered which, unlike the parental cells, are no longer capable of utilizing L-glutamic acid but require L-glutamine for growth. The change appears to be heritable in that cell progeny continued to be glutamine-requiring, although occasional nonrequiring colonies did occur. In order to further establish that the change of the nutritional requirement is due to a genetic mutation rather than to phenotypic adaptation, a fluctuation test on the occurrence of spontaneous reversions was performed. Prior to this test, the mutant clone was purified by eliminating the accumulated spontaneous revertants. The results of the test indicate that reverse mutations indeed occurred randomly at this locus. The rate of reverse mutation to prototrophy was estimated as 2.8×10^{-7} per cell per generation.

Cytological examination established the clonal nature of these mutational isolates as well as the origin of revertants. Furthermore, several mutants were found to possess characteristic chromosome marker(s), which might or might not be related to the mutational event, but which do provide additional cellular phenotypes.[3]

Resistance to 8-Azaguanine (AG^S). – The hamster cells are sensitive to 8-azaguanine at concentrations greater than 1 μg/ml. By mass selection, a cell subline was derived which exhibited a 1000-fold increase in resistance to the drug as compared with the parental cells. The resistant subline consists of a single, uniform karyotype. Further experimental data support the conclusion that resistance is due to a randomly occurring spontaneous mutational event and not to adaptation. Resistance arises from a loss or reduction of the inosinic acid pyrophosphorylase activity with resultant inability of the cell to utilize preformed purines or their analogs. At this locus, both forward and reverse mutation rates can be estimated when cells are grown in appropriate media. The spontaneous forward mutation rate to drug resistance was 6×10^{-7}, and the reversion rate was about 10^{-7} per cell per generation. Furthermore, there is evidence for the occurrence of a series of partial revertants with varying capacities of purine utilization. This last finding was confirmed by cell-free assay of the enzyme activity in parental as well as mutant cells.

Somatic Cell Hybrid. – Finally, we have succeeded in making somatic cell hybrids through fusion by plating the glutamine-requiring cells and azaguanine-resistant cells together in an experimental medium in which neither parental cell could survive alone. A number of morphologically distinct colonies were observed. One such colony was isolated which was shown to contain the combined chromosome complements from both parents. This tetraploid hybrid clone can be propagated through apparently normal mitosis. Functional tests indicate that both glutamine auxotrophy and azaguanine resistance behave as recessive characters in the hybrid cell.[4]

Discussion. – Because of the aneuploid nature of the parental line, it was possible that each locus studied might have existed in a hemizygous state due to the loss of the homologous chromosome or a deletion of the chromosome segment bearing its allele. An alternative possibility is that the parental cell was heterozygous at both loci at the time of their selection.

We have shown here the feasibility of mutation selection as well as genetic analysis through the use of the cell-fusion technique. In a following report[5] we present evidence that both forward and reverse mutations can be induced with a chemical mutagen in these hamster cells. We are hopeful that these studies will be extended and that the techniques we have been developing in this model system would be applicable to human cells in culture. Indeed, this is one of our immediate planned projects.

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26.15 MAMMALIAN CELL GENETICS. II. CHEMICAL MUTAGENESIS

E. H. Y. Chu H. V. Malling

Experimental induction of gene mutations in mammalian somatic cells has not been previously demonstrated. Only negative results were reported[1] when systematic attempts were made to induce mutations by various physical and chemical agents in a human bone marrow cell line. We have isolated, from populations of Chinese hamster lung cells, mutant clones which are either auxotrophic for L-glutamine (gln^-) or resistant (AG^S) to 8-azaguanine.[2] Our recent experiments clearly demonstrate that ethyl methanesulfonate (EMS) significantly increases the mutation frequencies at both loci.

The possible background of spontaneously occurring mutations was removed by treating the cells in appropriate selection media prior to induction experiments. After mutagenic treatment of the cells, selective agents were added at various times to the cultures: (1) for recovering reversions from gln^- to gln^+, medium lacking glutamine but containing glutamic acid was used; (2) for recovering the forward mutations from the azaguanine sensitivity (AG^S) to resistance (AG^S), 9 to 30 μg/ml of the drug was added to the medium; (3) for recovering reversions from AG^S to AG^S, the selective medium contained aminopterin, hypoxanthine, thymidine, and glycine. Both the survival and mutation frequency were determined on the basis of the frequencies of colonies formed.

Preliminary experiments established the cytotoxicity of the mutagen in the hamster cells. EMS at 0.01 *M* for 2 hr killed 30 to 40% cells and induced a tenfold increase of reversions per survivor to glutamine prototrophy. The same EMS treatment raised the forward- and reverse-mutation frequencies concerning the drug resistance three- to sevenfold. After 4 hr treatment, the surviving fraction was reduced to 10% but gave an even greater increase of mutation frequencies at both loci. The above induced frequencies also did not include those of partial or "leaky" revertants. Furthermore, we found the following factors affect the mutation yield: (1) conditions of cells before treatment, (2) inoculum size, and (3) time between mutagen quenching and the addition of selective agent (mutation expression). Figure 26.15.1 shows a significant increase in the frequency of induced mutations from AG^S to AG^S as the mutation expression time increases. The spontaneous mutation frequency remains the same during this period. Microscopic examination revealed that there had been three successive cell divisions during the same period. The average cell generation time was 12 hr, but the first cell division after mutagen treatment was delayed 6 hr because of the handling of the cultures. Since each cell progeny stays within a limited area, the multiplication in cell number within each colony does not affect the absolute number of colonies. The demonstrated increase in the mutation frequency is thus a real one.

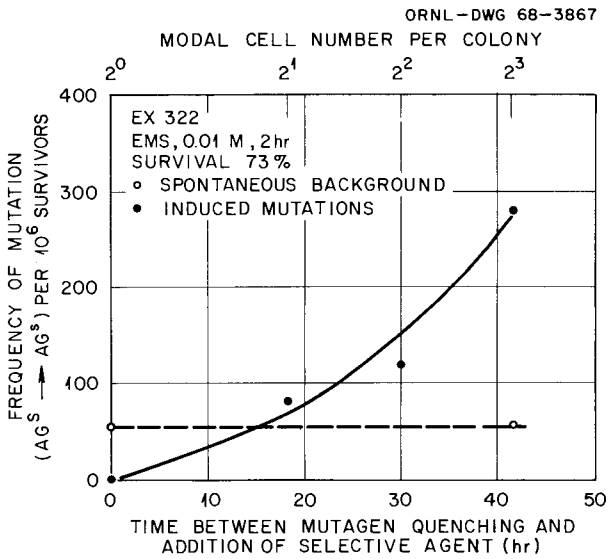

Fig. 26.15.1. The Change in the Frequency of 8-Azaguanine-Resistant Mutations in Chinese Hamster Lung Cells Induced by Ethyl Methanesulfonate as a Function of Posttreatment Expression Time.

Experiments are under way to maximize the frequency of EMS-induced mutations and to test other chemical and physical mutagens on these and other loci in the hamster cells.

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26.16 POSTTREATMENT BY STREPTOMYCIN AND MUTATION BY MNNG IN *PARAMECIUM*

R. F. Kimball E. G. Bailiff
Stella W. Perdue

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) produces mainly base pair substitution mutants.[1] In this respect it differs from the mutagens – x rays, ultraviolet radiation, and alkylating agents – previously used in our studies of repair of premutational damage in *Paramecium*. This previous work showed that exposure to streptomycin after mutagenic treatment but before S decreases mutation yield, whereas no effect is found when the streptomycin exposure begins after S. The interpretation was made that streptomycin decreases the mutation yield by delaying S, thus allowing more time for repair of premutational damage.[2]

Attempts to apply this test for reparable premutational damage to MNNG-treated paramecia have given variable results when the streptomycin exposure was begun immediately after the MNNG treatment, and the overall difference was not significant (Table 26.16.1). Exposure to streptomycin 24 hr after MNNG also produced no effect. In a number of experiments, however, a small but consistent *increase* in mutation yield was found when the streptomycin exposure was begun about $2\frac{1}{2}$ hr after the MNNG treatment, and the overall difference was significant at the 1% level.

The results show that MNNG does indeed produce reparable premutational damage, since the mutation yield can be altered by postmutagen treatment. Microspectrophotometric measurements suggested that the nuclei in MNNG-treated cells often underwent replication during the delayed streptomycin treatment. It is possible that the conversion of premutational damage to final mutation is enhanced when streptomycin is present during replication. We are planning to examine this possibility more carefully with other mutagens and to explore the reason for the variable results when streptomycin exposure is begun immediately after MNNG treatment. In this way we hope to find out whether MNNG is really basically unlike the other mutagens in its behavior.

References

[1]H. V. Malling and F. J. de Serres, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, pp. 48–50.

[2]R. F. Kimball, *J. Cell. Comp. Physiol.* **58**, suppl. 1, 163 (1961).

Table 26.16.1. Effect of Streptomycin on MNNG Induced Mutation

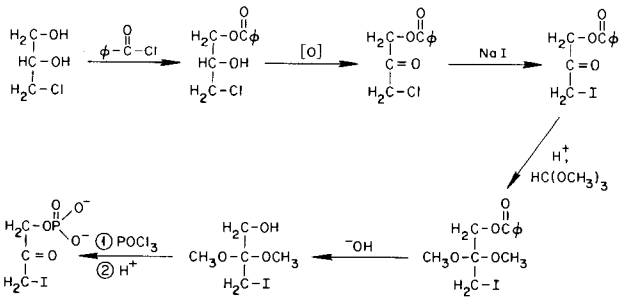
Streptomycin Exposure (time after MNNG treatment)	Mutation Yield (*M* units)	P_{diff}	Range of Differences
Immediately	0.73	>0.05	+0.48 to –0.31
None	0.60		
2.5 hr	1.05	<0.01	+0.52 to +0.28
None	0.67		
24 hr	0.66	>0.05	+0.11 to +0.02
None	0.60		

26.17 CHEMICAL MODIFICATION OF PROTEINS

F. C. Hartman

A Heavy-Atom Derivative of Rubredoxin. – Highly purified rubredoxin from *C. past.* has been amidinated with ^{14}C-labeled α-(*p*-iodophenyl)-acetimidate in an attempt to obtain a heavy-atom derivative suitable for x-ray crystallographic studies. Reaction conditions have been established which result in the incorporation of approximately one mole of reagent per mole of protein. Comparisons of the uv and visible spectra of native and amidinated rubredoxin indicate that no major conformational changes have accompanied the modification. The amidinated rubredoxin has been crystallized, and preliminary characterization experiments suggest that the modification has occurred at the N-terminal amino group.

A Potential Active Site Reagent for Aldolase, Triose Phosphate Isomerase, and Glycerol-l-Phosphate Dehydrogenase. – Since dihydroxy acetone phosphate (DHAP) is a substrate of aldolase, triose phosphate isomerase, and glycerol-l-phosphate dehydrogenase, a protein reagent structurally related to DHAP could perhaps be employed to label the active sites of all three enzymes. To test this hypothesis, 1-hydroxy-3-iodo-2-propanone phosphate (HIPP), a potential alkylating reagent resembling DHAP, has been synthesized, and its reaction with the above enzymes studied. HIPP was prepared by the following sequence of reactions:



HIPP inactivates the enzymes under consideration. Initial experiments on the kinetics of the aldolase inactivation are consistent with a modification of the DHAP binding site.

26.18 INSTRUMENTATION FOR X-RAY DIFFRACTION

J. R. Einstein

Magnetic Tape Facility. – A magnetic tape input-output facility was attached to the PDP-8 computer, which is the directing component of the automated diffractometer. All programs used for crystal setup and for data collection have been modified to allow their use as part of the "Dectape" library system. With this system it is now possible to read any program from magnetic tape into the computer memory in seconds, instead of up to 10 min, as required by reading from punched paper tape, the standard input medium. The magnetic tape system will later be used for automatic linking of programs, thus adding a very large "slow memory" to the computer.

Cooling Device for the Diffractometer. – Design and construction of a cooling device for the diffractometer have been continued. The purpose of the device is to maintain crystal specimens at a finely controlled low temperature by means of a stream of cold nitrogen gas. The delivery tube, to be mounted on the diffractometer, and incorporating a thermistor sensing element and a small heater for fine control, has been designed and is under construction. Components for an electronic controller have been assembled. The work has been done with the cooperation of Bio-engineering.

26.19 STRUCTURE OF A PHOTODIMER OF 1-METHYLTHYMINE

J. R. Einstein C. H. Wei

The molecular structure of a photodimer of 1-methylthymine has been determined by x-ray diffraction methods. The photoproduct, obtained by irradiation of frozen solutions of 1-methylthymine, was furnished to us by J. L. Hosszu, J. W. Longworth, and R. O. Rahn of this Division. The crystals are monoclinic, with $a = 7.542 \pm 0.001$, $b = 6.228 \pm 0.001$, $c = 14.463 \pm 0.0015$ A, $\beta = 117.04 \pm 0.01°$, space group $P2_1/c$, with two dimers per unit cell. Hence, the dimer molecules are centrosymmetric, and can correspond to none but isomer IV (*trans-anti*) of Wulff and Fraenkel.[1] Three-dimensional x-ray data were collected on the Oak Ridge Computer-Controlled Diffractometer.

The structure was solved by the symbolic addition procedure with the use of the computer program MAGIC.[2] Refinement was by full-matrix least squares. Based on 1048 independent reflections, a value of 5% has been obtained for the reliability index $R_1(F)$. The cyclobutane ring is planar and is approximately square, with bond lengths of 1.543 and 1.571 A (e.s.d. 0.005 A) and bond angles of 90.4 and 89.6° (e.s.d. 0.2°). All other bond lengths and angles fall within normal ranges. The pyrimidine rings, having lost the $C_5 - C_6$ double bonds present in the monomer, are not nearly so planar as in methylthymine or in thymine, but have a maximum deviation of 0.14 A out of the least-squares "best" plane. The dihedral angle between (normals to) the pyrimidine "best" plane and the cyclobutane plane is 54°.

References

[1]D. L. Wulff and G. Fraenkel, *Biochim. Biophys. Acta* **51**, 332 (1961).

[2]E. B. Fleischer, A. L. Stone, and R. B. K. Dewar, *MAGIC – Multiphase Automatic Generation from Intensities in Centric Crystals*, FORTRAN program, University of Chicago, 1966.

26.20 PREPARATION AND PRELIMINARY X-RAY DATA ON THYMINE DIMER POTASSIUM SALT

C. H. Wei

It is known that there are four different stereoisomers[1] of thymine photodimers. As a continuation of our previous work on the structure of isomer IV (*trans-anti*) of the analog 1-methyl thymine dimer,[2] we are interested in obtaining an unambiguous structure for isomer I (*cis-syn*) of thymine dimer, since this is the isomer obtained by uv irradiation of DNA. This isomer can also be obtained by irradiating frozen aqueous solutions of thymine. Samples of thymine dimer I, prepared by the latter method, were purified by column chromatography.[3]

Since attempts to obtain suitable single crystals of the dimer were unsuccessful, the potassium and sodium salts were prepared and recrystallized. A large number of optically acceptable crystals were examined by x-ray diffraction photographs before crystallographically suitable crystals were found. Crystals of thymine dimer potassium salt, $(C_5H_5N_2O_2K)_2$, are monoclinic with lattice parameters $a = 7.01$ A, $b = 26.17$ A, $c = 8.02$ A, and $\beta = 99.0°$. The observed density of 1.52 g/cm^3 (obtained by flotation) is in good agreement with the calculated value of 1.50 g/cm^3 corresponding to four formula units in a unit cell. The systematic absences are $\{hkl\}$ for $(h + k)$ odd and $\{h0l\}$ for l odd, indicating probably space groups $C2/_c$ and C_c.

We intend to collect a complete three-dimensional set of counter data and to determine the structure.

Investigation of the sodium salt is also in progress.

References

[1]D. L. Wulff and G. Fraenkel, *Biochim. Biophys. Acta* **51**, 332 (1961).

[2]J. R. Einstein *et al.*, *Chem. Commun.* 1063 (1967). The structure of isomer IV of thymine dimer has also been solved by x-ray diffraction; cf. N. Camerman, S. C. Nyburg, and D. Weinblum, *Tetrahedron Letters* **42**, 4127 (1967).

[3]Thanks are due to J. E. Donnellan, Jr., and R. S. Stafford of this Division for carrying out this purification.

26.21 X-RAY CRYSTAL STRUCTURE DETERMINATIONS OF TWO TRANSITION-METAL COMPLEXES

C. H. Wei

The determination of crystal structures by x-ray diffraction is occasionally hindered by unusual phenomena: the formation of twinned crystals and the statistically disordered nature of the compound in the crystalline state.

Structure of Twinned $Rh_4(CO)_{12}$. – A three-dimensional x-ray investigation of twinned orthorhombic crystals of $Rh_4(CO)_{12}$ has been completed. The successful structure analysis was accomplished by the application of simple derived mathematical relations,[1] which enabled a breakdown of the observed F^2 data for the twin composite into the F coefficients for the single-crystal twin component. The results of the three-dimensional refinement of the structure conclusively indicated (1) that the individual twin component possesses a centrosymmetric monoclinic unit cell with $a = 9.25$ A, $b =$

12.02 A, c = 17.74 A, $\beta = 90°$, and symmetry $P2_1/c$ and (2) that the twinning mirror plane parallel to the unique *b* axis and perpendicular to either the *a* or c axis results in pseudoorthorhombic D_{2h}-*mmm* Laue symmetry.

This is the first known case in which a crystalline structure with an incoherent twinning mechanism has been unambigously determined by the application of simple derived equations[1,2] utilizing a complete set of three-dimensional data. This method of treating a twinned crystal should be applicable to other similar cases.

Structure of Disordered $Co_6(CO)_{16}C_2S_3$. – The reaction of $Co_2(CO)_8$ with carbon disulfide was first reported by Marko *et al.*[3] to give a polynuclear cobalt carbonyl sulfur complex $Co_4(CO)_{10}CS_2$. The reaction has been reproduced in this laboratory, but chemical analyses of the product suggested an empirical formula $[Co_2(CO)_5CS]_n$, where $n = 3$ was found from a molecular-weight determination in $CHCl_3$ solution.[4]

A three-dimensional set of counter data, using the sample kindly furnished to us by Drs. Bor and Marko of Hungary, was collected on an automated diffractometer[5] in collaboration with P. A. Agron of the Chemistry Division of ORNL. Results of x-ray analysis showed that the correct molecular formula is $Co_6(CO)_{16}C_2S_3$, which is in agreement with the chemical analyses to within experimental error. The crystals contain four molecules in an orthorhombic unit cell of symmetry *Pnma* and of dimensions *a* = 17.67 A, *b* = 13.074 A, c = 13.493 A. The molecular configuration of $Co_6(CO)_{16}C_2S_3$ consists of two different trinuclear moieties, $CCo_3(CO)_9$ and $SCo_3(CO)_7SC$, linked together via a bridging sulfur atom by two tetrahedral-type S – C bonds. All molecular parameters are not unlike those found for other similar polynuclear cobalt carbonyl sulfur complexes. The most unusual feature of this structure is that each molecule possesses disordered orientations on both sides of a crystallographic mirror plane (m_b), which extremely complicated the analysis. The structure has been refined by full-matrix least squares[6] to a final unweighted *R* value of 5.9% (based on F^2) for 938 observed reflections.

This structure determination has provided another example of an unambiguous identification of molecular formula by x-ray crystallography and has also revealed an unusual type of crystalline disorder distinctively different from those found for $Co_4(CO)_{12}$[7] and $Fe_3(CO)_{12}$.[7]

References

[1]C. H. Wei, G. R. Wilkes, and L. F. Dahl, *J. Am. Chem. Soc.* **89**, 4792 (1967).

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[3]L. Marko, G. Bor, and E. Klumpp, *Angew. Chem.* **75**, 248 (1963).

[4]Galbraith Laboratory, Inc., 2323 Sycamore Dr., Knoxville, Tenn. 37921.

[5]We are indebted to Dr. H. A. Levy of the Chemistry Division of ORNL for his permission to use the Oak Ridge Computer-Controlled X-Ray Diffractometer and his assistance in using this instrument.

[6]The least-squares computer program ORFLS (by W. R. Busing, K. O. Martin, and H. A. Levy, Chemistry Division, ORNL) has been modified by Dr. J. R. Einstein of this Division so that individual atoms could be assigned either isotropic or anisotropic thermal parameters for the refinement.

[7]C. H. Wei and L. F. Dahl, *J. Am. Chem. Soc.* **88**, 1821 (1966).

26.22 SINGLET AND TRIPLET ENERGY TRANSFERS IN L-TRYPTOPHANYL-L-TYROSINE AND RELATED HETEROPOLYPEPTIDES

J. W. Longworth R. O. Rahn
J. A. Knopp

The characteristics of energy transfer between the residues of polypeptides may be obtained from measurements of fluorescence and phosphorescence yields and excitation and emission spectra and from ESR measurements.

The dipeptide L-trp-L-tyr at 25°C and pH 7 has a fluorescence predominantly from the tryptophanyl residues, with a peak at 340 nm, even when the excitation wavelength is chosen at the peak of the tyrosyl absorption. The tyrosyl contribution to the fluorescence spectrum can be separated from that of tryptophan, since tryptophan both absorbs and fluoresces to longer wavelengths than tyrosine. The tyrosyl spectrum has a peak at 325 nm and a quantum yield of 5% of the tryptophan quantum yield. The fluorescence excitation spectrum of the tryptophanyl fluorescence was measured at 380 nm, as tyrosyl residues have no appreciable fluorescence at this wavelength. This excitation spectrum has, in addition to the tryptophan absorption, a tyrosyl absorption contribution. Hence, energy absorbed by tyrosyl

residues is transferred to tryptophanyl residues, which then fluoresce. At 77°K, the tryptophanyl and tyrosyl residues have the same quantum yield of fluorescence, though there is still singlet energy transfer from tyrosine to tryptophan. However, there is no tyrosyl phosphorescence. Since the tyrosyl triplet state lies at a higher energy than that of tryptophan, a triplet transfer from tyrosyl to tryptophanyl residues probably takes place.

At pH 13, the fluorescence at 25°C or at 77°K is from tyrosinate residues only; there is no tryptophan contribution detectable. Since the fluorescence excitation spectrum at pH 13 is identical to the absorption spectrum, there must be complete singlet energy transfer from tryptophan to tyrosinate. An unusual situation prevails for the triplets, however, as now tryptophan has a lower-energy triplet than tyrosinate. The dipeptide phosphorescence at pH 13 is pure tryptophanyl emission, even for excitation at 305 nm. This latter wavelength only excites tyrosinate residues, yet the phosphorescence is still only tryptophanyl phosphorescence. Therefore, there is complete triplet-to-triplet transfer from tyrosinate to tryptophan.

The phosphorescence observations are entirely substantiated by separate triplet ESR measurements. In the ESR technique, it is found that tyrosine, tyrosinate, and tryptophan are easily separately measured in a mixture. For the dipeptide, no evidence is found for a tyrosyl triplet at pH 7 or a tyrosinate triplet at pH 13; the only triplet observed at wavelengths of excitation is that of tryptophan.

Analogous fluorescence, phosphorescence, and ESR observations have been made with two heteropolypeptides, poly-L-trp-L-tyr – (1:1) and (1:4). No tyrosine fluorescence or phosphorescence is found at 25°C or 77°K in both polymers at pH 7. Fluorescence titration studies at 25°C show that a single tyrosinate residue quenches 16 tryptophanyl residues in the (1:1) polymer and 6 residues in the (1:4) polymer. The magnitude of the fluorescence quenching suggests that tryptophan-to-tryptophan singlet transfer may also occur. At pH 13, the (1:4) polymer has a fluorescence only from tyrosinate and a phosphorescence from tryptophan, even when the excitation is at 305 nm. Triplet transfer is a short-range phenomenon that can only take place between neighbors, and of necessity some tyrosinate triplets cannot have tryptophan neighbors. Then there must be tyrosinate-to-tyrosinate triplet transfers in this polymer.

We have evidence for electronic energy transfers of the following kind. For singlet-to-singlet transfers: tyrosine to tryptophan and tryptophan to tryptophan. For the triplet-to-triplet transfers: tyrosine to tryptophan, tyrosinate to tryptophan, and tyrosinate to tyrosinate.

26.23 SINGLET ENERGY TRANSFER IN RIBONUCLEASE

J. W. Longworth Maria Del Carmen Battista
J. A. Knopp

Extensive studies in this laboratory with several model systems have shown that there is a transfer of electronic energy absorbed by tyrosyl residues to tyrosinate residues (ionized tyrosyl) at the singlet level. Energy transfer has been proposed by several authors to account for the alkaline fluorescence titration behavior of the tyrosyl fluorescence of RNase. However, the fluorescence of the acceptor tyrosinate residues was not reported, and no conclusive evidence can be gained simply from a titration study of the donor. In this study the solvent used was ethylene glycol, since in this solvent all the tyrosyl residues of RNase titrated normally and the absorption at 300 nm gives directly the percentage ionization of the tyrosyl residues.

The fluorescence of the tyrosyl residues was measured as a function of the percentage ionization of these residues, and it was found that tyrosinate residues partially quenched the tyrosyl fluorescence. When the excitation wavelength was that of an isosbestic point, an isoemissive point was observed in the fluorescence spectral titration. There can only be an isoemissive point if there is sensitization of the tyrosinate by absorption in tyrosyl. A more direct proof of sensitization is to determine the excitation spectrum of tyrosinate fluorescence, but because of the low quantum yield no conclusive experiment has been achieved to date.

RNase in water exhibits a biphasic pH titration of the tyrosyl groups, and the absorption spectra do not have an isosbestos for approximately the second half of the tyrosyl titration. Absorption spectral measurements do not give the percentage of tyrosyl ionized, because there is also absorption from cysteinyl anions. However, the cysteinyl anion contributes only 20% of extinction at the end of the tyrosyl titration, so that it is possible to approxi-

mate the percentage of tyrosyl ionization by neglecting the cysteinyl effect. The quenching determined from the fluorescence titration of RNase in water was found to be twice that found in glycol, so there must be a greater extent of transfer in water than in glycol. The fact that all the tyrosyl residues titrate normally in glycol is usually taken to mean that RNase in glycol has an open conformation, in which all functional groups are available to the solvent, compared with the compact conformation found in water. Thus the fluorescence titration data indicate that the extent of energy transfer depends on the conformation of the protein.

To determine the presence of energy transfer between identical residues, the degree of polarization of the fluorescence was measured. Transfers between like molecules which have different orientation to each other cause a depolarization. Such depolarizations have been observed by other workers with RNase in propylene glycol at pH 7 and at low temperatures. We have confirmed these observations at pH 7 and extended them to pH 13, where all the tyrosyl residues are ionized. In RNase at pH 13 a small depolarization is found compared to *N*-acetyl-L-tyrosinamide, indicating transfers between tyrosinate residues.

We have found evidence for singlet transfer in RNase involving tyrosine to tyrosine, tyrosine to tyrosinate, and tyrosinate to tyrosinate.

26.24 ENERGY TRANSFER IN POLY-L-TYROSINE: FLUORESCENCE POLARIZATION STUDIES

J. A. Knopp J. W. Longworth

Fluorescence at 25°C and fluorescence, phosphorescence, and electron spin resonance at 77°K have been used to study electronic energy transfer in poly-L-tyrosine. All these experiments have shown that the ionization of a single tyrosine residue in the polymer results in the quenching of the fluorescence of 30 to 50 neutral tyrosine residues by energy transfer. Since there cannot be as many nearest neighbors to a single residue, considerable energy transfer between neutral tyrosine residues must occur to explain the high quenching efficiency. To determine if neutral tyrosine-tyrosine transfer does take place, the fluorescence polarization of poly-L-tyrosine was examined.

N-Acetyl-L-tyrosine amide and poly-L-tyrosine – D. P. of 140 – were dissolved in two solvent systems, ethylene glycol:water:dimethyl sulfoxide and propylene glycol. The polarization of fluorescence was measured with an instrument similar to that used by Weber and Bablouzian.[1]

To determine the extent of energy transfer from fluorescence polarization measurements, all molecular rotations must be prevented so that the limiting polarization is observed. Hence, polarization of fluorescence of *N*-acetyl-L-tyrosine amide in propylene glycol was measured as a function of temperature, and at temperatures less than −60°C, the polarization reached a constant value. The polarization of fluorescence of both tyrosine monomer and polymer at −60°C was determined as a function of wavelength of excitation. The resultant spectrum for *N*-acetyl-L-tyrosine was identical to that published by other workers. The degree of polarization at 285-nm excitation was 0.36. However, the values for 285-nm excitation of poly-L-tyrosine were much lower, 0.06 in propylene glycol and 0.10 in ethylene glycol:water:dimethyl sulfoxide.

In absence of energy transfer, we expect that the intrinsic polarization of tyrosine residues in poly-L-tyrosine will be the same as for *N*-acetyl-L-tyrosine amide. The large decrease in going from the monomer to the polymer is indicative of the presence of energy transfer between neutral tyrosine residues. This result is in qualitative agreement with the quenching studies.

The expected depolarization due to energy transfer can be calculated if the orientations of the residues and the probabilities of transfer to different neighbors are known. As the α helix is the most probable configuration, simple calculations were performed to determine the depolarization as a function of the number of energy transfers. Comparison with the experimental results did not give a unique answer. Further refinements of these calculations should permit us to determine if there is quantitative agreement between quenching and polarization measurements and to gain some insight into the nature and extent of the migration of excited states in such helices.

Reference

[1] G. Weber and B. Bablouzian, *J. Biol. Chem.* **241**, 2558 (1966).

26.25 EXCIPLEX FORMATION IN MODEL SYSTEMS AND PROTEINS

J. A. Knopp J. W. Longworth

N-Acetyl-L-tryptophan methyl ester (ATME) in 2,2,4-trimethylpentane has a fluorescence spectrum at 298°K which is the mirror image of the absorption spectrum and has no Stokes shift. Addition of 1% 2-propanol to the system does not alter the absorption spectrum but converts the fluorescence emission to an entirely different spectrum, which is now structureless and peaked at 335 nm. The implication is that within the excited-state lifetime of the singlet state of ATME, a complex is formed with 2-propanol, that is, "exciplex" formation.

The fluorescence of tryptophan and many globular proteins at 298°K in polar solvents is characterized as a structureless band peaked at 340 nm. In polar glasses at 77°K the maximum shifts to 315 nm, and the emission spectrum has vibrational structure for both tryptophan and protein tryptophanyl residues. Thus, proteins at 298°K fluoresce as the tryptophan exciplex, and the complex formation is prevented at 77°K.

As a result we expect that there will be a protein with a fluorescence spectrum at 298°K which is peaked between 300 and 320 nm and has vibrational structure, that is, has no exciplex formation at 298°K. The fluorescence of the coat protein of TMV particles behaves in this manner; its fluorescence spectrum is structured and peaks at 320 nm at both 298 and 77°K. Exciplex formation takes place when the virus and coat protein are denatured by dissolving the virus into dimethyl sulfoxide/1,2-ethanediol (1:1 v:v); at 298°K, the fluorescence spectrum peaks at 340 nm and at 320 nm at 77°K. Thus the formation of an excited-state complex between the excited singlet state of tryptophanyl residues and a neighboring polar group occurs in the majority of proteins at 298°K but not at 77°K.

26.26 FURTHER TALES OF TYROSINE

Maria Del Carmen Battista J. W. Longworth

Crammer and Neuberger in 1943, before the advent of photoelectric absorption spectrometers, showed that (1) ionized tyrosyl residues of egg albumin and insulin absorb to longer wavelengths than the neutral form, (2) the maximum absorption reached is time dependent, and (3) there is no intersection of neutral and ionized spectra for insulin. D. Shugar in 1951, using the then new photoelectric spectrophotometers, showed that the absorption spectrum of fully ionized ribonuclease, like insulin, was greater than that of the neutral molecules at all wavelengths, but that the two spectra intersected during the first half of the titration. In 1962 Sage and Singer found that ribonuclease in ethylene glycol had isosbestic points for the entire titration range. The cause of the extra and time-dependent absorption by ribonuclease, in alkaline aqueous solutions at pH > 11, had remained unexplained until December 1967.

A dimeric dipeptide, L-tyrosine-*bis*-L-cystinyl dimethyl ester, was found to behave identically, in its absorption spectrum, to ribonuclease and insulin. For pH > 11 in water there were no isosbestic points; but in ethylene glycol there were clearly defined ones. Thus the extra absorption is produced when a disulfide linkage is adjacent to tyrosyl residues. Confirmation of this interpretation is provided by the polypeptide oxytocin, in which the one tyrosyl residue is adjacent to the single disulfide bridge. Oxytocin has no isosbestic points in water but has them in ethylene glycol. In contrast the amino acid L-cystine, in equimolar mixture with *N*-acetyl-L-tyrosine amide, maintains an isosbestos behavior for some 90% of the tyrosyl titration, and is thus quite distinct from L-tyrosyl-*bis*-L-cystinyl ester.

Donovan[1] reached an identical conclusion, namely, that the cause of the extra absorption is the disulfide linkage. He demonstrated that there is a titration of the disulfide by alkali, leading to the production of cysteinyl anion; this anion absorbs strongly at 235 nm, but still has appreciable absorption to 300 nm.

Reference

[1] J. W. Donovan, *Biochem. Biophys. Res. Commun.* **29**, 734 (1967).

26.27 GENETIC ANALYSIS OF HYDROXYLAMINE-INDUCED PURPLE MUTANTS (*ad-3*) IN *NEUROSPORA CRASSA*

H. V. Malling F. J. de Serres

Hydroxylamine (HA) has a mutagenic effect in many microorganisms (bacteriophage, bacteria, yeast, and *Neurospora*). The mutagenic specificity

of HA depends on the conditions under which the treatment is done. In prokaryotes with double-stranded DNA, HA induces almost exclusively base-pair transitions from guanine-cytosine (GC) to adenine-thymine (AT) when the concentration of HA is higher than $>0.1\ M$. At low concentrations ($10^{-3}\ M$), HA has only slight mutagenic effect but is highly toxic, and under these conditions can induce chromosome damage in mammalian cells.

Hydroxylamine is readily oxidized to very reactive compounds; this oxidation is inhibited by using high concentrations of HA. Many of the possible oxidation products of HA are known to be toxic, and Freese and Freese[1] have proposed that the toxicity of low concentrations of HA is due to an accumulation of these oxidation products. This is also likely to explain the chromosome damage induced by low concentrations of HA. A priori one would not expect that mammalian cells can survive treatment with 1 *M* HA and 3 *M* NaCl, which is the necessary condition for obtaining high specificity of HA.

We feel that it is important to study the mutagenic action of HA in a eukaryotic system where both chromosome damage and point mutations can be detected, and the genetic alterations identified at the molecular level.

In the dikaryon system of *Neurospora crassa*,[2] the following biological parameters can be studied: (1) nuclear and cytoplasmatic inactivation, (2) frequency of recessive lethal mutations over the entire genome, (3) frequency of chromosomal deletions in the *ad-3* region, (4) frequency of point mutations in the two structural genes *ad-3A* and *ad-3B*, and (5) identification of the genetic alteration at the molecular level.

Conidia from a genetic balance heterokaryon were treated for various lengths of time (4 to 12 hr) with 1 *M* HA dissolved in 3 *M* NaCl solution and then adjusted to pH 6.2. In this experiment we found:

1. No difference between the survival of the heterokaryotic fraction of the conidia and the total population of conidia for the various times of treatment by HA. This indicates that the inactivation is most likely cytoplasmatic and not nuclear.
2. The mutation frequency of *ad-3* mutants increases proportional to the square of the treatment time. This indicates that two different processes, both increasing in proportion with the treatment time, are necessary for induction of mutations with HA in *Neurospora*. HA reacts predominantly with cytosine in the DNA; either one or two HA molecules can react with one cytosine molecule. If the chemical changes of cytosine which can lead to a mutation require reaction with two HA molecules, it provides an explanation for the two-hit kinetics of the mutation induction curve.
3. Genetic analysis (Table 26.27.1) of the mutants by means of heterokaryon tests for complementation showed that the frequency of *ad-3A* and *ad-3B* mutations increases in proportion to the square of treatment time. The ratio of *ad-3B* to *ad-3A* mutants is 1.72. Among the HA-induced *ad-3B* mutants 64% were complementing, 52% with nonpolarized and 12% with polarized complementation patterns; the remaining 36% were noncomplementing. Only 1% of the *ad-3* mutants were too leaky to test.
4. The frequency of recessive lethal mutations (Table 26.27.1) in the entire genome (not including the *ad-3* region) increases with longer treatment time, but the numbers are too small to determine the kinetics of the induction. The frequency of chromosomal deletions in the *ad-3* region does not increase with longer treatment time and is not significantly different from the expected spontaneous mutation frequency of this type of mutations. Therefore, HA does not induce chromosome deletions.

We can conclude therefore that when a eukaryote (*Neurospora*) is treated with HA under conditions which give the highest specificity for reaction with cytosine in the DNA, only point mutations are induced.

Among NA-induced *ad-3B* mutants with a base-pair transition at the mutant site, 77% had a nonpolarized complementation pattern, 7% a polarized complementation pattern, and 16% did not complement.[3] Frequencies of each of these three classes among HA-induced mutants were, respectively, 52%, 12%, and 36%. The nonsense triplets in *E. coli* are UAA, UAG, and UGA; the analogous codons and anticodons in the DNA are, respectively, ATT/TAA, ATC/TAG, and TCA/AGT. From this we can see that nonsense triplets cannot be induced by an AT → GC transition, and they cannot revert by a GC → AT transition. It would therefore be expected that mutagens which predominantly induce GC → AT transitions should give rise to a higher frequency of mutants with polarized complementation patterns and noncomplementing mutants than mutagens which pre-

Table 26.27.1. The Frequency of the Different Types of Mutations Induced by HA in the *ad-3* Region of *Neurospora crassa*

Hours Treatment	Mutation Frequency per 10^6 Survivors									
	Forward-Mutation Frequency (directly determined)	*ad-3A*				*ad-3B*				Leaky
		$ad\text{-}3A^R$	$ad\text{-}3A^R$ + RL	Total $ad\text{-}3A^R$	$ad\text{-}3A^{IR}$	$ad\text{-}3B^R$	$ad\text{-}3B^R$ + RL	Total $ad\text{-}3B^R$	$ad\text{-}3B^{IR}$	
0	0.64	0	0	0	0	0.33	0	0	0	0
4	1.27	0.76	0	0.76	0	0.51	0	0.51	0	0
6	7.31	2.9	0.26	3.2	0	4.2	0	4.2	0	0
8	9.62	3.0	0	3.0	0	6.4	0	6.4	0.18	0
10	18.4	5.6	0	5.6	0	11.6	1.03	12.6	0	0.38
12	27.4	9.4	1.2	10.6	0	14.3	1.64	15.9	0.41	0.41

dominantly induce AT → GC transitions. By analysis of the genetic alteration in NA-induced *ad-3B* mutants at the molecular level, we found that the majority had GC at the mutant site.

The difference between the frequency of the different types of complementation patterns among the NA-induced *ad-3B* mutants and the HA-induced *ad-3B* mutants fits well to these expectations. The relatively simple test for allelic complementation seems, therefore, to have diagnostic capability for determination of the type of genetic alterations in samples of *ad-3B* mutants induced by different mutagens.

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26.28 THE PHOTODYNAMIC EFFECT OF CARCINOGENIC POLYCYCLIC HYDROCARBONS IN *NEUROSPORA CRASSA* AND CHINESE HAMSTER CELLS

H. V. Malling E. H. Y. Chu
F. J. de Serres

A positive and statistically significant association between photodynamic and carcinogenic activities of polycyclic hydrocarbons has been demonstrated in protozoa such as *Paramecium caudatum.*[1] Several other lines of evidence further indicate a promotive effect of black light on the induction of cancer by polycyclic hydrocarbons, although these compounds alone can also induce cancer in many tissues and organs that have never been exposed to light. Two possibilities exist: (1) that the light enhances those actions of the polycyclic hydrocarbons that lead to cancer induction or (2) that it induces an entirely new set of reactions in the cells which may or may not cause cancer. The following experimental results seem to favor the first possibility: 3,4-Benzpyrene (3,4-BP) is a strong carcinogen which, after being painted on mouse skin, binds covalently to the skin DNA. For a series of different polycyclic hydrocarbons, there is a strong correlation between their reaction with DNA in the mouse skin and the ability to induce cancer.[2] When mixed with isolated DNA, 3,4-BP binds physically to the DNA, but when the complex is irradiated with black light, 3,4-BP or its photodynamic degradation products bind covalently to the DNA.[3] Allison and Mallucci[4] found that in mammalian cell cultures, polycyclic hydrocarbons preferentially accumulate in the lysosomes, leading to the destruction of their membranes and thereby liberating the enzyme content of the lysosomes into the cell. The disruption of lysosomes by polycyclic hydrocarbons is enhanced drastically by irradiation with black light. Since one of the enzymes in the lysosome is a DNAase which has been shown to attack the chromosomes, it is possible that this attack may be the first step toward carcinogenesis.

Since the possibility exists that the study of the photodynamic effect of polycyclic hydrocarbons may elucidate the general biological activities of these compounds, and hopefully also the carcinogenic effect, we felt that it was important to study this effect in systems where certain specific biological questions could be answered. For this study we used two systems:

1. the *Neurospora* dikaryon system, where we can study: (1) nuclear and cytoplasmic inactivation, (2) frequency of chromosomal deletions in the *ad-3* region, (3) frequency of point mutations in the two structural genes *ad-3A* and *ad-3B*, and (4) identification of the genetic alteration at the molecular level;
2. somatic cell cultures of Chinese hamster, where we can study: (1) cell death, (2) frequency of chromosomal aberrations, (3) rupture of the lysosome, and (4) induction of mutations.

By using the two systems concurrently and under treatment conditions which are as similar as possible we want to see how far we can extrapolate the data from *Neurospora*, in which a detailed genetic analysis of the mutants can be done, to the cultured mammalian cells, in which direct cytological examinations and mutagenesis studies are feasible.

Two closely related but different benzypyrenes exist: a 3,4-BP and a 1,2-BP. 3,4-BP is carcinogenic and 1,2-BP is not. If there is any correlation between photodynamic toxicity and the carcinogenic activity of polycyclic hydrocarbons, then only 3,4-BP should show photodynamic toxicity. In both systems this is in fact what we found (Fig. 26.28.1). In cell cultures, 3,4-BP at the concentration used

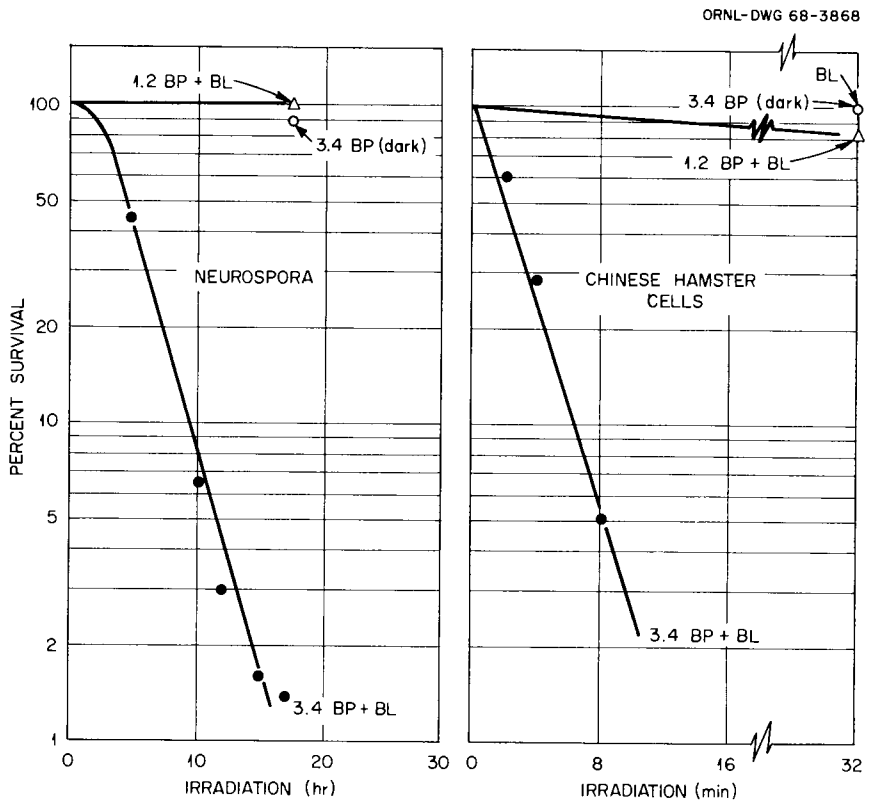

Fig. 26.28.1. Effect of 1.2-BP or 3.4-BP Treatment in Dark Or Under Irradiation with Black Light on the Survival of the Heterokaryotic Fraction of Conidia from *Neurospora* Heterokaryon 12 and of Chinese Hamster Cells. Both the conidia and cells were treated with 10 γ/ml of the polycyclic hydrocarbons dissolved in 1% dimethylformamide solution or only in the solution alone. A General Electric fluorescence tube (F15T8 BL) was used as a source of black light.

was also slightly toxic without irradiation. In *Neurospora* no difference was found between the survival of the heterokaryotic fraction and of all conidia, indicating that the inactivation of 3,4-BP plus black light was probably due to cytoplasmic damage rather than nuclear destruction. The experiments will be extended to include other polycyclic hydrocarbons as well as to study the type of cytogenetic alterations in both systems that may be induced by these compounds in combination with black light.

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26.29 DEVELOPMENT OF SELECTIVE SYSTEMS FOR SOMATIC CELL HYBRIDIZATION

James D. Regan Susumu Takeda
W. H. Lee

The technique of somatic cell hybridization, now documented by numerous literature reports, offers a new approach to somatic cell genetics.

Our studies have basically two approaches:

1. Myxovirous (Sendai)-induced hybridization of human diploid, nonneoplastic fibroblasts (WI-38)

with minimal deviation tumor cells. Such tumor cells have a potentially useful genetic marker in that they are inducible for tyrosine transaminase by steroid hormones. Thus one can study the behavior of this marker in the presence of a normal human genome. Such hybrid cells can also be useful in studying other aspects of normal vs tumor cells.

2. Development of selective systems for the recovery of spontaneous hybrid cells. In particular, the study is directed toward the hybridization of lower-vertebrate cells with mammalian cells. We have already shown that these lower-vertebrate cells are photoreactivable and contain photoreactivating enzyme.[1] Mammalian cells in vitro do not exhibit photoreactivation.[2]

For these selection experiments we have developed the following cell systems:

1. Thymidine kinase-lacking iguana heart (IH) cells. These cells were selected using BUdR treatment combined with visible light irradiation.[3] Such cells are unable to grow in medium containing aminopterin, hypoxanthine, and thymidine (the "HAT" medium of Szybalski and Szybalski).[4]

2. Physiological conditions for WI-38 human diploid fibroblasts and for tumorogenic mouse fibroblasts such that the cells are unable to utilize galactose as a carbon source.

Mating experiments are conducted in HAT medium with galactose substituted for glucose.

For example:

IH (TK^-, Gal^+) × WI-38 (TK^+, Gal^-)

on

HAT-galactose medium

↓

IH, WI-38 hybrid (TK^+/TK^-, Gal^+/Gal^-) .

Analysis of cells derived from such experiments is in progress.

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26.30 SYNCHRONIZATION OF LOWER-VERTEBRATE CELLS BY THE ANTINEOPLASTIC AGENT HYDROXYUREA

Susumu Takeda James D. Regan

Hydroxyurea (HU) is known to inhibit experimental tumors in mice[1] and chronic granulocytic leukemia in man.[2] It is an effective inhibitor of DNA synthesis in mammalian cells in vitro.[3] Hydroxyurea is believed to act by inhibition of ribonucleotide reduction[3] and is lethal to cells in DNA synthesis while preventing non-"S" cells from proceeding into S.[4]

We undertook to investigate the effect of HU on established lower-vertebrate cell lines because we wished to synchronize these cells for several experimental purposes, in particular for studies of photoreactivation in the cell cycle.

Established cell lines from marine fish (GF) or from freshwater fish (FHM) and a clonally derived amphibian cell (HO8W2) have been used in these studies.

Figure 26.30.1 shows the amount of synchrony of DNA synthesis induced by treating HO8W2 cells with various concentrations of HU for 25 hr.

The highest peak of DNA synthesis after removing the drug was achieved with 0.1 m*M* HU. However, this concentration produced only about 50% inhibition of DNA synthesis during the treatment. A concentration of 1.0 m*M* HU reduced DNA synthesis to about 1% of that of controls. However, the lower (and later) peak of DNA synthesis and the data on mitotic index at this concentration (Fig. 26.30.2) indicate that there was considerable irreversible cell damage during HU treatment. Thus, with HO8W2 cells, partial synchrony with a sharp peak of DNA synthesis and subsequently of mitosis can be achieved with 0.5 m*M* HU. For a greater degree of synchrony with minimal cell damage 0.1 m*M* HU appears to be the concentration of choice (Figs. 26.30.1 and 26.30.2).

GF cells have appeared to be more sensitive to HU. Concentrations of 0.5 m*M* seemed, by the above criteria, to produce considerable cell damage in treatment periods of about three-fourths the generation time of the cell line, which is 44 hr.

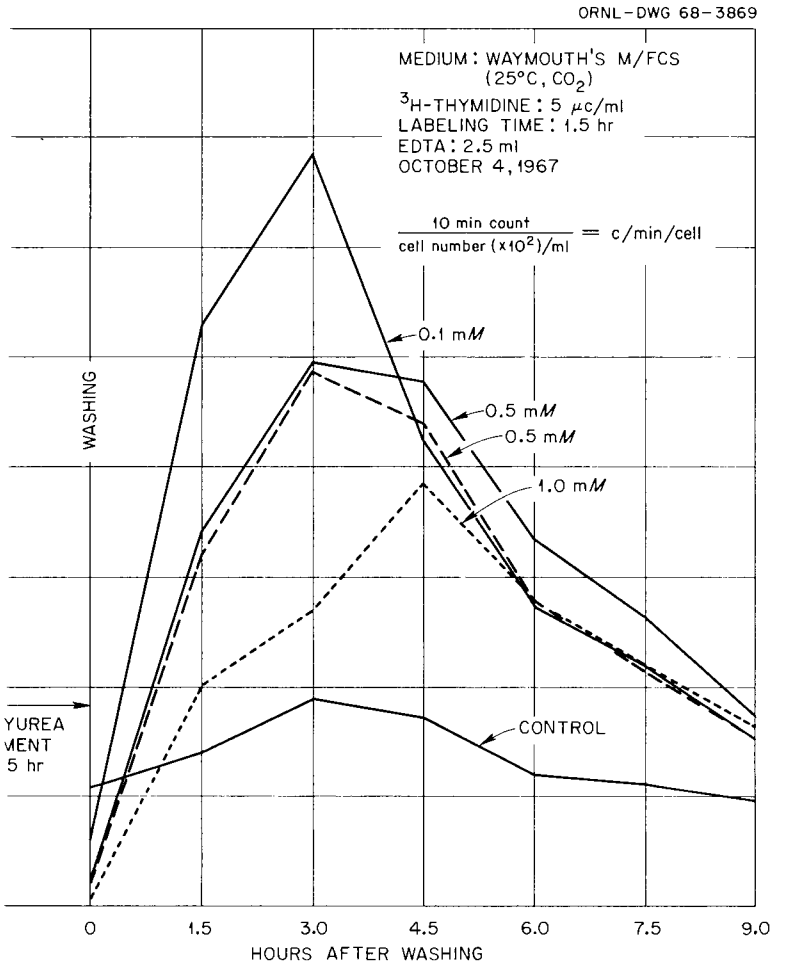

Fig. 26.30.1. Effect of 25 hr of Treatment with Various HU Concentrations on DNA Synthesis in H08W2 Cells. DNA synthesis was assayed by the method of Bollum. HU was removed at time zero.

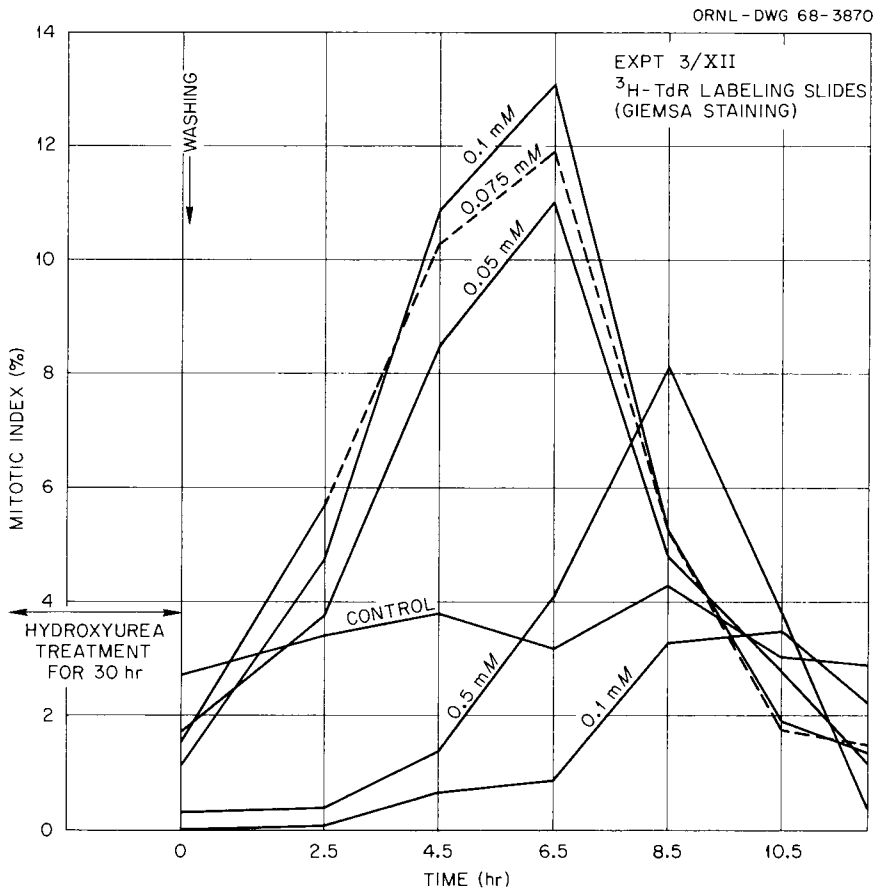

Fig. 26.30.2. Effect of 30 hr of Treatment with Various Concentrations of HU on Mitotic Index in H08W2 Cells. HU was removed at time zero.

FHM cells, in contrast, seemed less sensitive than either GF or HO8W2 to HU. A concentration of 1.0 m*M* appeared best for maximal inhibition of DNA synthesis during treatment, maximal synchrony upon release, and minimal cell damage.

The data from all these cell lines indicate they are more sensitive to HU than human cells, where 2.5 m*M* HU is used for synchronization.[4] FHM cells appear similar to Chinese hamster cells, where 1.0 m*M* is the concentration of choice for inducing synchrony.[5] GF and HO8W2 cells are, however, more sensitive to the action of HU than either human or Chinese hamster cells.

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26.31 INHALATION CARCINOGENESIS

D. G. Doherty
J. O. Brick
M. G. Hanna, Jr.
Alfred Hellman
Paul Nettesheim
R. F. Newell

The protocol and experimental techniques for the inhalation experiment have been described in previous reports in this series. In the past year the installation of two additional chambers has been

completed, and the chambers have been calibrated. The exposure of two new experimental groups of mice to a calcium chromate dust that has been reported to produce lung tumors on implantation has been initiated. This dust is some 60 times as soluble as chromic oxide and nearly twice as toxic to the mice when given via the inhalation route. The total number of C57Bl/6 mice for all groups has risen to 7547, of which 645 have been sacrificed for initial or group terminal examination and 2329 have been found dead. The treatment and cumulative exposure time for all groups is given in Table 26.31.1. With few exceptions all dead animals have been subjected to gross autopsy, the major organs fixed, and histological sections examined. The gross autopsy findings are presented in Table 26.31.2.

A few general comments on the experiment can be made at this time. In contrast to earlier reported inhalation experiments with smog, where the median death time was about 16 months and all the animals were dead by 20 months, the median death time for

Table 26.31.1. Chamber Operations Through December 31, 1967

Chamber No.	Pretreatment	Chemical	Exposure (hr)
I	½ irradiated[a]	0	2635
II	½ irradiated[a]	Cr_2O_3, 52 ppm 27 ppm	2466 363
III	½ irradiated[a]	Ozone, 1 ppm Gasoline, 30 ppm	2726
IV	½ irradiated,[a] all virus	Gasoline, 30 ppm	2419
V	½ irradiated,[a] all virus	Cr_2O_3, 53 ppm 27 ppm	1955 363
VI	½ irradiated,[a] all virus	0	2163
VII[b]	0	45 pans	0
VIII[b]	Irradiated	45 pans	0
IX[b]	Virus	45 pans	0
X[b]	Irradiated, all virus	45 pans	0
XI[c]	½ irradiated[a]	45 pans	0
XII[c]	½ irradiated,[a] all virus	45 pans	0
XIII	½ irradiated,[a] all virus	$CaCrO_4 \cdot H_2O$, 35 ppm	730
XIV	½ irradiated[a]	$CaCrO_4 \cdot H_2O$, 35 ppm	817

[a]One-half the animals in each chamber were irradiated where so indicated.

[b]No exposure in the chambers; kept in pans to compare with chambers I and VI controls.

[c]No exposure in chambers; kept in wire cages to compare with chambers I, VI, VII, VIII, IX, and X controls.

Table 26.31.2. Gross Autopsy Diagnosis

Chamber Starting Date	I 1-18-66	II 11-15-65	III 12-13-65	IV 2-28-66	V 4-5-66	VI 4-29-66	VII 3-8-66	VIII 4-11-66	IX 6-24-66	X 7-14-66	XI 9-8-66	XII 9-16-66	XIII 6-8-67	XIV 5-16-67
Hydrocephalus	2		1	1	1		1	2						
Thymoma	1	2	3	1						1				
Starvation (long teeth)	3	4	2	4	1	2	2	3	2		2	2		
Wasting (cause unknown)	3	5		1		1								
Physical injury (handling, etc.)	1	15	2			1	1		1	1	1			1
Dehydration	2													
Urogenital disease	138	111	132	113	126	113	6	7	4	2	27	21		2
Gastrointestinal disease	1	6	1	11	10	10		1	1	3	1	6	1	
Respiratory disease	3	6	3	4	5	3	4		4	3	1	4	1	
Neoplasia	26	27	21	24	20	9	18	25	13	10	3	6		
Myocarditis		4	2	1	1		4	3	2	4	1			
Undetermined	48	79	49	29	40	35	41	56	27	28	18	24	12	9
Other (dermatis, etc.)	58	106	81	55	83	68	17	28	14	14	77	31		4
Total Deaths in Chambers	286	365	297	244	287	242	94	125	68	66	131	94	14	16

our SPF mice in the chambers will be over 20 months regardless of treatment. To date, all mice have remained free from such bacterial pathogens as peudomonas and salmonella as well as endo and ecto parasites and some seven mouse viruses that can be typed for at present. This longer life-span for all animals should enhance the chance for tumor induction. It is plain that the mortality in all groups (except XIII and XIV, Table 26.31.2), when compared at an equal age = 16 months, is greater for those mice living on wire as opposed to the pan controls, whose median death time will probably be in excess of 25 months. The stress of living on wire is primarily reflected in death from urogenital disease in the males and hair loss in the females. This hair loss has seemingly little or no effect on mortality in the females. The radiation and virus pretreatment clearly results in an accelerated mortality in the males that is independent of chemical treatment when compared at 16 or 19 months. Paradoxically, the reverse effect is seen in the females, the pretreated animal deaths being lower in all groups at these time periods.

The pathological findings indicate that the PR8 influenza virus causes a severe, often widespread, pneumonia, which leaves behind a chronic lesion in the lung parenchyma. This lesion is characterized by pseudoglandular transformation of the lung alveoli and focal fibrosis, which persists for the life-span of the animal. The other agents used in this study (calcium chromate, ozonized gasoline, and x irradiation) do not produce any immediate histologically identifiable lesions. The insoluble chromic oxide dust reaching the lung alveoli is phagocytized rapidly by the alveolar cells. Most of the compound is transported out of the lung as these phagocytic cells leave the alveolar spaces via the tracheobronchial tree. No histologically identifiable tissue damage is produced by the chromic oxide dust. The lung tumors observed to date are all typical pulmonary adenomas and adenocarcinomas, the highest incidence being in the ozonized-gasoline-treated groups compared with the others at similar exposure times. The most frequent pathologic lesions found in sites other than the lung are urogenital disease, dermatitis, leukemias (lymphocytic and reticulum cell), polyarteritis, glomerulosclerosis, ovarian tumors, and hepatomas. The relationship between the frequency of these lesions and the various applied treatments remains to be determined.

It is obvious that large quantities of data are being generated in this study, which includes 20 different treatments, which, with both sexes, yields 40 experimental groups. In order to adequately handle this material it has all been programmed for automatic record storage, retrieval, and analysis. Included in the current programs are chamber operating conditions, animal assignment, animal weights, death records, histology number, and gross and micro pathology. An open pathology dictionary, handled in word form and computer translated to numeric form, is used in this latter program. All programs have incorporated numerous error checks and are designed to facilitate the analysis of all possible interactions. Preliminary analysis of some of the early results is currently under way as a test of the systems.

26.32 COMPUTER PROGRAMS USED IN THE INHALATION CARCINOGENESIS PROJECT

M. G. Hanna, Jr. J. O. Brick
B. S. Bishop

The inhalation carcinogenesis experiment involves over 7000 experimental mice which were derived from a barrier maintained, specific pathogen-free breeding colony. Due to the complexity of manual records on the breeding colony and the experimental animals, and the laborious process involved in retrieving pertinent information from such records, computerized programs were developed for these studies.

The computerized breeding system consists of five major programs written in the COBOL programming language for an IBM 7090 computer. The programs are code named MATCAR, SUMCAR, MASCAR, REMCAR, and MATGEN. The MATCAR program maintains the master file on all breeding mice. In general this program will (1) create a new master record for mated mice; (2) record all daily events of the breeding colony; (3) delete incorrect records from the master tape; (4) produce printed schedules of coming events, that is, wean, mate, retire; (5) print cage cards necessary for proper identification; and (6) make error analyses of the input data. The SUMCAR program provides a summary of animals that are not weaned and animals in holding cages to be mated. The primary use of this program is the advance information of the maximum possible number of weanling mice obtainable in a given period of time. The MASCAR program is used to obtain a printed list of the breeder records on the master tape. When the

breeding master tape acquires additional breeder records with each update run, it is desirable to remove inactive records to a separate storage tape. The REMCAR program is designed to accomplish this removal. The MATGEN program is essentially the same program as MATCAR with the exception of the input data format.

The animals are assigned to experimental cages when they are weaned at three weeks of age. These cages are placed on the EXHAN master tape. The EXHAN computer program is designed to create and update a master tape containing records of animals used in the carcinogenesis experiment. The experimental cage is first assigned to a test chamber. If the animals are not assigned to a test chamber by the time they are twelve weeks old, program DEXHAN removes the record from the master tape. Records maintained on EXHAN are death transactions, cage weight transactions, and pathology records. The pathology section of the master record is updated from program PATHAN. This program has been created to reduce the alphabetic pathology data to an eight digit code using the SNOPCO dictionary tape, and recording the code in the experimental master tape originally created by EXHAN. The final program ANNEX resorts the pathology information and provides output in the form of listings or graphic plots. These pathology data can be called for by chambers, sex, cause of death, as well as specified time intervals.

26.33 SERUM ALPHA GLOBULIN FRACTION: SURVIVAL-AND-RECOVERY EFFECT IN IRRADIATED MICE

M. G. Hanna, Jr. Paul Nettesheim
W. D. Fisher

The concept of humoral factors promoting radiation recovery has been the subject of extensive investigations in the past. The interest in such factors was supported by observations of slight but significant enhanced survival and/or hemopoietic recovery in animals injected with serum, plasma, or serum components shortly after irradiation. In recent immunologic studies we observed an enhanced hemopoietic recovery in the spleen exposed to internal radiation of ^{125}I which was bound to isologous alpha-macroglobulin (19S). This observation prompted studies on the effect of various alpha-macroglobulin preparations on radiation survival and hemopoietic recovery. Results demonstrated that isologous and heterologous macroglobulin preparations significantly enhanced radiation survival and accelerated the recovery rate of hemopoietic tissue.

Macroglobulins were prepared by centrifuging 25 to 30 ml of whole serum on a 1000-ml sucrose density gradient in either the B-IV or B-XV zonal ultracentrifuge. The gradient was monitored at 260 and 280 mμ as it passed through a modified Beckman B spectrophotometer. The separation showed two peaks. The gradient was collected in 40-ml fractions. After four to six runs were made, material from the second peak was pooled and concentrated. Analytical ultracentrifugation showed that it contained approximately 50% 19S protein (about 750,000 mol. wt). An ultracentrifugally homogeneous 19S fraction was obtained by a second centrifugal separation in the B-IV zonal rotor. The 19S fractions were characterized as the alpha complex by acetate strip and acrylamide gel electrophoresis.

Initially we studied survival of mice after whole-body irradiation. Random groups of 12- to 13-week-old $BC3F_1$ male mice were irradiated with a cumulative dose of 750 r x irradiation. The cumulative 30-day mortality from seven separate experiments (10 to 35 mice per group) showed the following results: Group 1 consisted of 205 mice receiving 750 r with no treatment after irradiation. The cumulative mortality for this group was 83.4%. Group 2 consisted of 180 mice given 750 r, and within 2 hr after irradiation they were injected intravenously with 3 or 6 mg of protein from the first peak (7S). The cumulative mortality for this group was 75.3%. Group 3 was composed of mice given 750 r plus 3 or 6 mg of isologous macroglobulin (2nd peak). A total of 191 mice was used in this group. In the first two experiments these animals received 3 mg of purified macroglobulin (homogeneous 19S). The groups in the other experiments received 6 mg of the crude macroglobulin preparation (approximately 50% was 19S). There was no significant difference in survival between these two groups. The cumulative mortality for the seven different experiments was 31.9%. Thus, isologous macroglobulin caused a significant increase in survival after 750 r of x irradiation.

Histologic studies were made of spleens from unirradiated mice injected with 6 mg of the fraction containing isologous macroglobulin. There was no morphological effect in either spleen white

or red pulp during the first ten days in mice treated with macroglobulin.

Changes in spleen weight were measured in animals irradiated with 750 r, followed either by no injection or by intravenous injection of 6 mg of isologous first-peak material or 6 mg of second-peak material (isologous macroglobulin). At 15 days there was a twofold increase in the ratio of spleen weight to body weight (means) in mice treated with macroglobulin, as compared with mice injected with the fraction from the first peak. At 20 days after irradiation the ratio of spleen weight to body weight in macroglobulin-treated animals was approximately 50% above that of the uninjected and the first-peak injected controls. The early increase in spleen weight in the macroglobulin-treated mice was the result of hematopoietic recovery.

In order to determine the magnitude of the enhanced recovery after macroglobulin treatment, the colony-forming unit (CFU) assay was used, along with the measured incorporation of ^{59}Fe in the spleen and bone marrow of animals exposed to 400 r x irradiation. The results of these studies are shown in Fig. 26.33.1. It can be seen that both the CFU count and ^{59}Fe incorporation in both spleen and bone marrow were significantly enhanced in animals injected with isologous macroglobulin after irradiation, as compared with the first peak and uninjected controls. The actual CFU measurements in the bone marrow showed a threefold increase at day 4 and a sixfold increase at day 7 after 400 r x irradiation. In the spleen there was a fivefold difference at day 4 and a tenfold increase in CFU at day 7. These studies show that isologous macroglobulin significantly enhances the rate of hematopoietic recovery in bone marrow and spleen of $BC3F_1$ male mice irradiated with 400 r. Similar results could be obtained in $BC3F_1$ and $C3_1F_1$ mice using rat and BALB/c mouse and human macroglobulin.

Studies were performed to determine when, in relation to the x irradiation, the macroglobulin was most effective in promoting hematopoietic recovery. The results of these studies are shown in Table 26.33.1. The data show that varying the time of macroglobulin injection in relation to x irradiation had a marked effect on the recovery of spleen CFU. When given at 1 hr after irradiation, the recovery-enhancing effect was far greater than when given 24 hr before or 24 hr after irradiation. It can also be seen that when three injections of 3 mg were given over the 24-hr period following 400 r, the recovery-promoting effect of macroglobulin

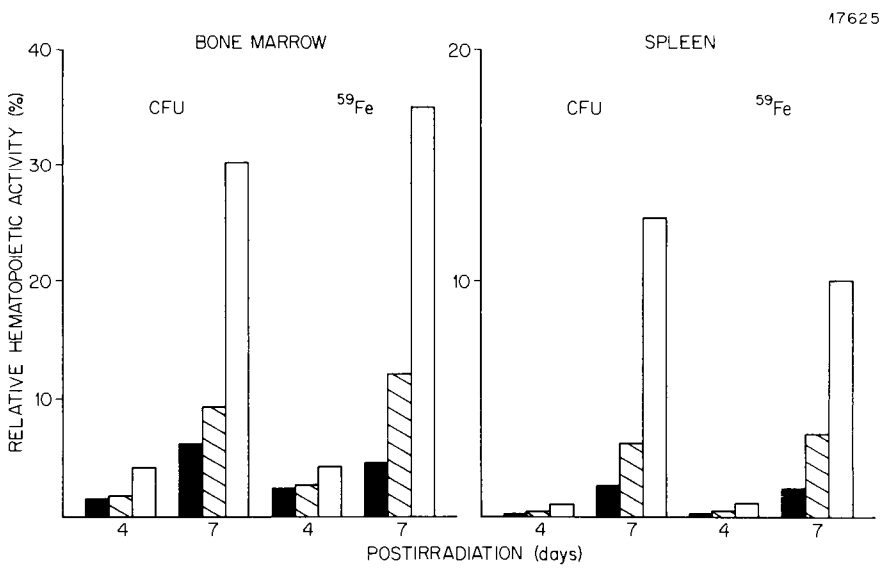

Fig. 26.33.1. Relative Hematopoietic Activity in Bone Marrow and Spleen, Four and Seven Days After 400-r X Irradiation, in BC_3F_1 Mice. Postirradiation treatment: ■ = none, ▨ = 6-mg first-peak fraction, □ = 6-mg second-peak (macroglobulin) fraction.

Table 26.33.1. Effect of Time of Injection and Repeated Injection of Isologous Macroglobulin on Hemopoietic Recovery of $BC3F_1$ Mice X Irradiated with 400 r

Treatment	Days After X Irradiation	Cell Source	No. of Recovered Donor Cells	Mean No. of Colonies[a] per Recipient Spleen	CFU per 10^6 Donor Cells	CFU per Spleen or Femur
			$\times 10^6$			
None	7	Spleen	36.8	0.54 ± 0.20	0.54	19.9
3 mg macroglobulin 24 hr before x ray	7	Spleen	46.4	1.58 ± 0.25	1.58	73.9
3 mg macroglobulin 1 hr after x ray	7	Spleen	49.8	5.67 ± 0.36	5.67	282.3
3 mg macroglobulin 24 hr after x ray	7	Spleen	38.3	1.73 ± 0.27	1.73	66.3
1 mg macroglobulin 1, 12, and 24 hr after x ray	7	Spleen	58.0	3.64 ± 0.51	3.64	211.2
3 mg macroglobulin 1, 12, and 24 hr after x ray	7	Spleen	57.3	6.36 ± 0.88	6.36	364.6

[a]Ten to twelve recipients per group; ±1 standard error of the mean.

was greater than when 3 mg was given in a single injection 1 hr after irradiation. It should also be noted that treatment with three times 3 mg of macroglobulin was more effective than three times 1 mg in promoting significant CFU recovery, indicating a dose response.

Several cytotoxic agents, such as colchicine, bee venom, and, in particular, bacterial endotoxin, have been shown to enhance radiation survival and/or hematopoietic recovery. However, these substances have been demonstrated to be optimally effective when given 24 hr prior to irradiation. In contrast, the macroglobulin fraction was found to be nontoxic in normal animals and has been shown to be most effective when given after x irradiation. This indicates that its mode of action is different from that of the above-mentioned agents. The consistency of the macroglobulin effect in the great number of experiments performed with various batches from different serum sources also seemed to rule out the possibility that we might be dealing with some form of a contaminant.

Bioassay for erythropoietin in polycythemic C3H mice showed that no detectable erythropoietin (greater than 0.05 unit) was present in 3 mg of either the first or second peak of $BC3F_1$ mouse serum protein preparations. Further study in which the first and second peaks were separated from serum isolated from phenylhydrazine-treated rats showed that the erythropoietin activity was entirely in the first peak. This rules out the erythropoietin as the active moiety measured in our studies.

The recovery-enhancing effect of serum macroglobulin in irradiated mice has proved to be highly reproducible. However, the mechanism by which the 19S alpha fraction acts on irradiated hematopoietic cells is still unknown. Future studies are planned to obtain information on the mechanism of action.

26.34 LOCALIZATION OF ^{125}I-LABELED ANTIGEN IN GERMINAL CENTERS OF MOUSE SPLEEN: EFFECTS OF COMPETITIVE INJECTION OF SPECIFIC OR NON-CROSS-REACTING ANTIGEN

M. G. Hanna, Jr. Mary W. Francis

The significance of persistence of antigen for production of specific antibody and maintenance of immunologic memory has been emphasized in

several reports. Recently the germinal centers (secondary follicles) of lymphatic tissue, which have long been considered to have a fundamental role in immune reactions, have been demonstrated to be sites of localization and persistence of antigen. This association has been described with a variety of antigens in lymphatic tissue of spleen and in lymph nodes. Interpretation of the role of the antigen–germinal-center association in immune reaction is limited by two unresolved questions: (1) the role of immunoglobulins in this process and (2) the immunologic specificity of the localization.

In order to further evaluate this question of specificity, it is necessary to determine whether the antigen–germinal-center association is a static phenomenon, in which a proportional amount of substance is nonspecifically trapped over a period of time and then slowly degraded, or a dynamic association involving a cycling of some form of the antigen through specific germinal centers only as long as this form is present in the circulation. Regarding the role of immunoglobulins in this process, no evidence has been presented as to which globulin or what structural features are recognized in the germinal centers.

Our approach to determining the specificity in this process was to study localization during the primary and secondary immune response as influenced by later competitive injection of specific and non-cross-reacting antigens.

Materials and Methods. – Male $BC3F_1$ mice 12 to 14 weeks of age were used. Human gamma globulin (HGG) labeled with ^{125}I was used as antigen. Bovine serum albumin (BSA) and sheep erythrocytes (SRBC) were used as competitive antigens.

Primary Response to HGG as Affected by Injection of Additional Substances on Third Day. – All animals were given 1.5 mg ^{125}I-HGG on day 0. Group 1 received no subsequent injection. Five groups of mice received one of the following injections on day 3: group 2, 1.5 mg HGG; group 3, 1.5 mg ^{125}I-HGG; group 4, 15.0 mg HGG; group 5, 1.5 mg SRBC; and group 6, 1.5 mg BSA. Autopsies were performed on four to five animals per group at serial intervals after injection of HGG. Tissue was taken for autoradiography and counting for radioactivity. Serum was prepared for passive hemagglutination.

Secondary Response to HGG as Affected by Injection of Additional Substances on Third Day. – Four of the experimental groups described above were repeated in this experiment. All animals were injected intravenously (iv) with 1.5 mg nonlabeled HGG on day 0. Group 1 received no subsequent injection; the remaining three groups received one of the following injections on day 3: group 2, 1.5 mg nonlabeled HGG; group 5, 1.5 mg SRBC; and group 6, 1.5 mg BSA. Five animals per group were killed at various postinjection intervals. The remaining animals in the four groups were injected iv with 1.5 mg ^{125}I-HGG. The same procedures were used as in the primary response studies.

Results and Discussion. – *The Role of Immunoglobulins in Antigen Localization.* – There is little question that antigen-antibody complexes injected into normal animals localize in germinal centers, or that localization of many antigens occurs more readily in the presence of specific antibody. However, the results of the present study show that the details of this localization are quite different from the primary response, primarily in length of persistence of antigen in germinal centers. Following secondary injection in animals with high serum antibody, enhanced deposition in germinal centers of antigen and/or antigen-antibody complexes was observed during the first few days. However, between three and five days a larger proportion of label was observed primarily in the lymphocyte mass of the nodule, where it was rapidly eliminated. During these intervals, little or no label was found in the spleen germinal centers. The functional significance in the immune reaction of the enhanced localization in passively immunized animals is questionable, since in these animals, in which follicular localization is enhanced, the primary immune potential is depressed.

To explain the overall results, we would speculate that localization of antigen in germinal centers is a dynamic process. For some antigens there are opsonins readily available at the time of injection which promote initial localization in germinal centers. These may be preformed immunoglobulins or some other specific factor. The continued localization of antigen over weeks or months, however, is a function of specific antibody and is highly dependent on the rate of production. A synchronous response would be in situations where antigen is given in the presence of serum antibody in passively or actively immunized animals where the persistence in germinal centers

is relatively short compared to the primary response. Further support for this idea might be obtained from the present study in comparisons of labeled and unlabeled germinal centers during the first 15 days of the primary response in groups 3 and 4. In mice injected initially with 1.5 mg ^{125}I-HGG and at three days with 15.0 mg unlabeled HGG, there is a significant decrease in the percentage of detectably labeled centers during the time of antibody production and de novo germinal center function. In mice receiving two injections of 1.5 mg ^{125}I-HGG, the percentage of labeled centers remained above 90% during the same time intervals. Thus, it might be expected that when the ratio of labeled to unlabeled HGG molecules was 10:1, in this proposed dynamic system the percentage of detectably labeled centers would decrease.

The Specificity of Antigen Localization in Germinal Centers. – The de novo formation of germinal centers to injection of different antigens on day 3 offers some information concerning the specificity of the antigen localization process. It was apparent from the studies with two labeled HGG injections that the enhanced germinal-center formation was in response to the second antigen injection. If the labeled HGG given in the first injection had been circulating among germinal centers in a nonspecific manner, there should have been an increase in the mean number of labeled centers in the 15-day interval following injection of BSA or SRBC on day 3. This was not the case; the numbers of labeled germinal centers in these treatment groups were not significantly different from those in animals receiving a single injection of HGG, although the total number of germinal centers was markedly increased. Thus, for some period of time germinal centers appear to be specific to the antigen which stimulated their development. The fact that ^{125}I-HGG was localized in preexisting germinal centers of the spleen indicates that this specificity is not permanent and that centers will eventually respond to a second or different antigen.

The Role of Germinal Centers in the Secondary Immune Response. – In comparison with mice given HGG alone, as measured both by serum antibody titer and by immune clearance, marked depression of the secondary immune potential occurred in mice given 1.5 mg of SRBC or BSA three days after the primary HGG injection. In the antibody titers measured during the primary response, there was no significant difference between these three treatment groups.

On the basis of the histologic data, it could be assumed that during the period of de novo formation of germinal centers in response to the second nonspecific antigen, there was a competition among germinal centers with different antigens, for progenitor cells functional in the secondary response. This competition occurred during the primary response at the time of development of the sensitized cell compartment.

The results of this study suggest that there may be a competition among germinal centers for the cells responsible for secondary immune response and that the antigen persisting in germinal centers may be functional in development of the secondary immunologic potential.

26.35 THE ULTRASTRUCTURE OF ANTIGEN LOCALIZATION AND VIRUS-LIKE PARTICLES IN MOUSE SPLEEN GERMINAL CENTERS

A. K. Szakal M. G. Hanna, Jr.

Lymphatic tissue germinal centers have long been considered to be functional in immune reactions and have recently been demonstrated to be sites of concentration and persistence of antigen. Several recent studies correlate the antigen in the centers with the presence of immunoglobulin. The purpose of the present fine-structure studies was to supplement the light microscopic autoradiographic observations made in this laboratory on the localization of soluble antigens in germinal centers of mouse spleen. Electron microscopic autoradiography was used in a systematic study of the first 30 days of the primary response in mice to ^{125}I human gamma globulin. With this technique we expected to more precisely determine the exact location of antigen in secondary lymphoid follicles and to clarify the ultrastructure of cells responsible for antigen retention.

BC3F$_1$ male mice were intravenously injected with 1 ml of 1.5 mg/ml of ^{125}I-labeled human gamma globulin (HGG). After injection the animals were killed at intervals of 1, 4, 7, 11, 15, 20, and 30 days. The excised spleens were bisected longitudinally and the two halves pre-fixed in 1% glutaraldehyde for 30 min to allow dissection of individual lymph nodules. Lymph nodules were then fixed in glutaraldehyde for another 90 min

and post-fixed in phosphate-buffered osmium tetroxide. Subsequently the tissues were dehydrated and embedded in epon. The specimens were double stained with uranyl acetate and lead citrate and prepared for electron microscopic autoradiography using Ilford L-4 nuclear research emulsion. The grids were then stored in the dark for ten weeks to obtain proper exposure of the emulsion. After exposure, the specimens were developed in Kodak Microdol-X, or in physical developer if higher resolution were desired. At least two-thirds of the available sections were examined in an HU 11 B Hitachi electron microscope.

Observation. – *Day 1.* – In the spleen white pulp, label was located intercellularly between lymphocytes. In the marginal zone and periphery of germinal centers, silver grains also occurred intercellularly but were occasionally found associated with cytoplasmic vacuoles and inclusion of phagocytic cells. Silver grains seen in the lymphatic nodules were associated primarily with two cell types: macrophages and reticular cells.

Day 4. – By day 4, silver grains were found mostly in the reticular cells of the germinal centers. Occasional grains still were seen intercellularly in the white and red pulp. Silver grains were also observed over the cytoplasm of small- and medium-sized lymphocytes. Reticular cells in germinal centers, associated with label at this interval, had a voluminous nucleus which contained deep clefts and a small distinct nucleolus. Their extensive cytoplasmic processes filled the intercellular spaces among the characteristic large cells (immunoblasts) of the germinal centers. The cytoplasm of the reticular cells was also filled with numerous folded membranes.

The membranous structure of the cytoplasm of the reticular cells was due to numerous infoldings of the plasma membrane. With detailed examination with the electron micrograph it was possible to follow the extensive plasma membrane along its convoluted course into the perinuclear region. These infoldings were unlike the structural changes seen in cells undergoing phagocytosis. Typical immunoblasts, grouped around the processes of the reticular cells, were characterized by a large, round, irregular nucleolus with peripherally located chromatin. Endoplasmic reticulum was infrequently found in these cells.

Day 7. – At the end of the first week, silver grains were found only in association with the large antigen-retaining reticular cells of the germinal center. The amount and the distinctness of the convolutions of the cell membrane infoldings of the reticular cells were increased in comparison with samples examined at earlier intervals (Fig. 26.35.1).

Days 15 to 30. – The relative position of silver grains in plasma membrane infoldings of reticular cells was unchanged. However, the number of silver grains in plasma membrane infoldings appeared to have decreased. By the end of the fourth week there were no detectable grains over the germinal centers in autoradiograms exposed for ten weeks, and the amount of plasma membrane infolding was slightly decreased. Plasma cells could be observed in the periphery of the germinal center after day 15.

During the examination of the specimens with the electron microscope, virus-like particles were frequently found in the germinal centers, always in the same areas as the silver grains. The particles measured 100 to 120 mμ and had a lighter outer and a darker inner shell with the highest electron density occurring between the shells. The central nuclear portion of the particles was only moderately electron dense and sometimes contained single eccentric electron-dense granules. These virus-like particles were primarily extracellular in a similar distribution as the labeled antigen and occasionally were connected to immunoblasts in the germinal center by a pedicle, giving the impression of budding.

Discussion. – During the course of this study, antigen was primarily retained extracellularly in the membranous infolding of reticular cells of the germinal centers. These infoldings of the plasma membrane, the occurrence of which appears to be antigen related, increased with time. At day 4, in occasional areas of the centers, the plasma membranes of these antigen-retaining reticular cells were closely associated with cytoplasmic processes of immunoblasts.

A proportionately large number of virus-like particles was observed in germinal centers compared with that seen in the associated lymphocyte mass of the primary nodules of the spleen. In the centers, the particles were located primarily extracellularly in infoldings of the antigen-retaining reticular cells. Occasionally, virus-like particles were also observed budding from typical immunoblasts. It is impossible to interpret the significance of these particles at this time. It is highly inter-

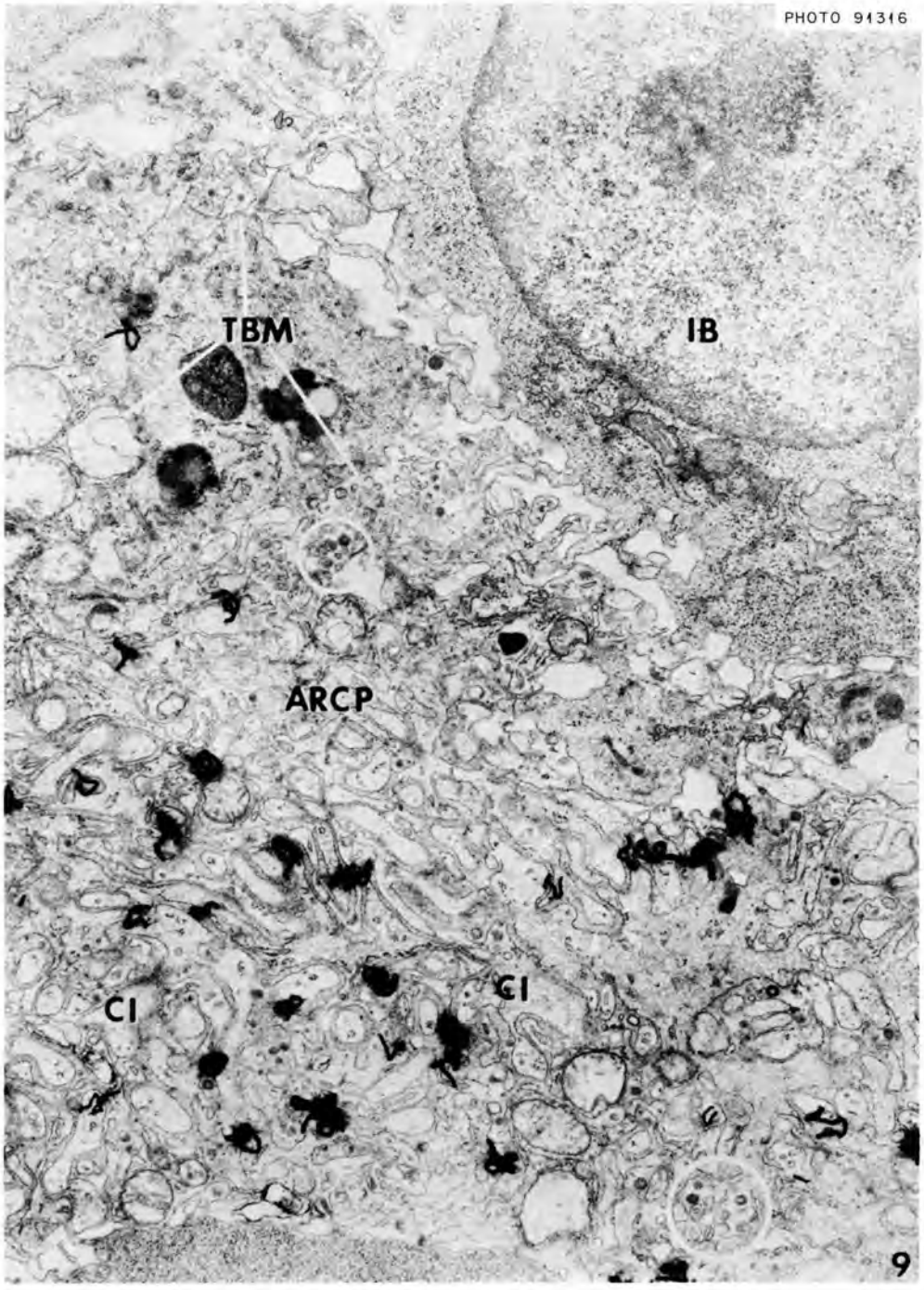

Fig. 26.35.1. A Microautoradiogram of a Small Area of a Germinal Center from the Lymphatic Nodule of Mouse Spleen Seven Days After the Injection of ^{125}I-Labeled HGG. The electron micrograph shows part of a process of an antigen-retaining reticular cell (ARCP) interdigitating with the villous processes of an immunoblast (IB). Observe the convoluted channels, formed by the plasma-membrane infoldings in the reticular cell process. Silver grains of the autoradiogram are located over the electron-dense material in the channels and never clearly over the cytoplasmic islands (CI) containing polysomes or swollen mitochondria. Note the piece of cytoplasm of a tingible body macrophage (TBM) without label and the numerous virus-like particles (circled) in the plasma-membrane infoldings. Epon, uranyl acetate, and lead citrate. 14,700×.

esting that the location of the majority of these particles was similar to that of the extraneous antigen. Also, this observation may be important in terms of the broader problem of indigenous latent murine virus, especially the murine leukemia virus.

26.36 EFFECT OF X IRRADIATION ON THE LOCALIZATION AND PERSISTENCE OF ANTIGEN

Paul Nettesheim M. G. Hanna, Jr.

Our studies concerning the recovery of secondary antibody-forming capacity from x irradiation suggested that persistence of antigen may play an important role in the recovery of the secondary antibody-forming potential. We therefore wanted to learn more about the effects of irradiation on the mechanism responsible for trapping and persistence of antigens in the lymphatic tissues. Recent studies had demonstrated that the phagocytic and degradative capacities of peritoneal phagocytes are resistant to supralethal levels of x irradiation. Human gamma globulin (HGG) labeled with ^{125}I was chosen as test antigen, since a considerable amount of information was previously obtained in our laboratory with this antigen. Mice were whole-body-irradiated with from 0 to 600 r either immediately before injection with labeled antigen or ten days after injection of labeled antigen. In a third group, x rays were given prior to an injection of *un*labeled HGG, and labeled HGG was injected 20 days later. The radioactivity in serum, liver, spleen, and kidneys was determined at intervals from 0 time up to 30 days. Autoradiograms were made of the various organs. The data from these experiments can be summarized as follows: x irradiation up to 600 r did not interfere with trapping of labeled HGG in germinal centers given in a primary injection; however, it caused enhanced clearance of the labeled antigen from the germinal centers regardless of whether x rays were given before or after the test antigen. When x rays were followed immediately by a primary injection of unlabeled HGG, then the initial trapping of a secondary injection of labeled test antigen given on day 20 after x ray was less efficient than that of unirradiated controls, and elimination of labeled antigen from the germinal center was enhanced. At no time did x rays affect the specific activity of liver, kidney, spleen, or serum in any of the described experiments. The data show that x irradiation, besides affecting the immune progenitor cell pool, also affects persistence of antigen (and under certain conditions efficiency of the trapping mechanism) in the germinal centers of the lymphatic tissues.

Therefore, in studies concerned with immunosuppression, the effect of the suppressing agent on the antigen trapping and retaining mechanism may be of major importance.

26.37 INHALATION CO-CARCINOGENESIS BARRIER FACILITY

J. O. Brick

Construction of the Inhalation Co-Carcinogenesis Barrier Facility was completed in June of 1966. The facility was designed and constructed for the purpose of producing specific-pathogen-free mice to be used in inhalation research. The initial mice transferred into the barrier were C57Bl/6 breeders that had been previously housed in Building 9720 under filter tops. The testing program indicated the mice had remained free of pigmented *Pseudomonas*, *Salmonella* and *Mycoplasma sp.*, internal and external parasites, and seven common murine viruses while in filter-top cages; therefore, all mice in the present barrier facility are housed in this manner. The testing program results, analyzed through September of 1967, are given in Table 26.37.1.

At the present time, in addition to the C57Bl/6J strain, a C_3Hf/Pi strain, $BL_6C_3F_1$ (C57BL/6 ♀ × C3Hf/Pi ♂) hybrids, and an inbred Fischer 344 rat strain are produced in the barrier. Experiments utilizing these strains are conducted in the environmentally controlled x-ray room and laboratory, both of which are located within the barrier. In this way, pathogen-free mice are produced, utilized in experiments, and housed under pathogen-free conditions, all within the same barrier area. Research of this nature has proven advantageous inasmuch as pathogen-free mice are clearly one answer to help eliminate the problem of mortality which is not directly related to the experimental outcome. In an effort to prevent undesirable contamination, daily procedures utilized within the facility are: (1) Personnel shower and wear special clothing; (2) feed, bedding, laundry, etc., are either steam- or gas-sterilized prior to barrier entry; (3) an air-handling system provides constant temperature and humidity and 12 to 15 air changes per hour; (4) cages, as well as bottles (filled with

Table 26.37.1. Results of Testing Program

Type of Sample	Total Number of Samples	Number Positive	Percent Positive	Positive for:
Fecal samples	6011	19	0.31	Nonpigmented *Pseudomonas* and pigmented *Pseudomonas*
Area samples	1311	12	0.91	Pigmented *Pseudomonas*
Object samples (cages, bottles, etc.)	758	165	20	Gram-positive cocci, gram-positive bacilli, gram-negative bacilli, and a variety of identified genera
Organ samples				
Weanling mice	496	2	0.4	Nonpigmented *Pseudomonas* and pigmented *Pseudomonas*
Retired breeders	770	0	0	
Serum samples	967	26	2.7	1.2% positive for Reo 3 and 1.5% positive for GDVII

14 to 16 ppm chlorinated water), are changed three times weekly; and (5) entry is restricted to animal caretakers and authorized research personnel.

26.38 FATE OF 1-(4-DIMETHYLAMINOBENZYLIDENE)INDENE IN RATS

C. T. Bahner D. G. Doherty

1-(4-Dimethylaminobenzylidene)indene (I) has been labeled in the methyl group with ^{14}C, or in the ring of the benzylidene moiety with ^{3}H, and used to study the metabolism of this antitumor agent. A single ip injection of 0.5 to 1.0 ml of arachis oil containing 8 mg of I per milliliter into young rats weighing 85 to 250 g was usually absorbed completely within 48 hr, but following repeated doses the yellow oil sometimes persisted several days after the last injection. Often a significant amount of unchanged I was found in the feces, and usually the abdominal fat was stained yellow by it. There was evidence of demethylation as one of the principal metabolic changes. Urea seemed to be one of the chief urinary metabolic products of ^{14}C-methyl-labeled I. Nearly half of the administered tritium was found in the urine and alcoholic extract of the feces within 48 hr, but only about one-seventh of the ^{14}C activity was accounted for in the same manner. Fecal excretion was much greater than urinary excretion in both cases. Autoradiograms of formalin-fixed tissues, including tumors, indicate that most of the radioactivity in the tissues is in forms which are removed during the fixing and paraffin impregnation, but a significant amount of radioactive material remains firmly bound to the tissues.

26.39 STUDIES ON IN VIVO PROTEIN PHOTOOXIDATION BY MONITORING TRITIUM LOSS FROM IN VIVO HISTIDINE MOIETIES

A. P. Harrison, Jr.[1]

In vitro protein photooxidation with methylene blue is known to involve preferential oxidation of the histidine moieties. To detect photodamage the protein was acid hydrolyzed; then assays for the various amino acids were undertaken by chemical and biological methods. These methods are laborious and inconvenient when many assays are required, as in cell survival-time studies. A rapid, sensitive test for in vivo protein photooxidation is described: Parallel cultures of *Escherichia coli* were labeled with tritiated amino acids. The cells were washed and suspended in 0.03 *M* sodium phosphate pH 7, and photosensitizer was added. Sus-

pensions were assayed for loss of tritium and viability after doses of illumination, and comparisons were made with suitable controls. Tritium lost from cells was detected in the supernatant after centrifugation or as loss from the pellet. More simple was to place 0.1 ml of formalized suspension directly on filter paper disks, dry the disks, and wash them with cold TCA. Tritium assays were taken with a liquid scintillation counter. The relative rates of in vivo tritium loss from protein amino acids were histidine > tryptophan > tyrosine > arginine and leucine. (No tritium loss was detectable with the latter two, and only a trace with tyrosine.) Histidine photodamage measured by loss of tritium correlated well with photodamage measured by means of microbiological assay on acid hydrolysates. Postlog cells lost histidine-tritium and viability less readily than log cells. Photoresistant mutants lost histidine-tritium and viability less readily than wild type.

Reference

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26.40 REGULATION OF ENZYME SYNTHESIS AND TURNOVER

F. T. Kenney Joseph Kendrick
L. E. Roberson

Regulation of enzyme levels is a fundamental part of orderly growth and differentiation; it is clear that a failure of dysfunction of this regulation may be fundamental to the characteristic disorganized growth of neoplasia. We are analyzing the mechanisms of regulation in normal liver in vivo and in cell cultures of minimal deviation tumors from rat liver. Insofar as these cultures respond to regulatory agents in a normal fashion, they offer a system in which the role of these agents can be studied individually and independently of the complex in vivo environment. On the other hand when instances are found in which the response of the hepatoma cells is lacking or abnormal, an alteration peculiar to the neoplastic state may have been uncovered. Such alterations might be particularly anticipated when hepatomas are formed as a result of treatment with carcinogens of the type which alkylate nucleic acids. We are seeking changes in regulatory mechanisms during the course of treatment with diethylnitrosamine; the pattern of ethylation of transfer RNA's due to this treatment will be analyzed in the Enzymology Group.

In mammalian tissues the amount of an enzyme protein can be changed by altering the rate of either the synthesis or the degradation of that protein. Our understanding of the mechanisms of degradation is very limited. We are studying molecular mechanisms of enzyme degradation in an attempt to analyze the basis for different turnover rates of various enzymes.

As reported previously,[1] in the steady-state or "basal" situation, tyrosine transaminase undergoes turnover with a half-life of about 1.5 hr. This rapid rate contrasts to a half-life of about three days for the bulk of the liver soluble proteins. When protein synthesis was experimentally blocked (by puromycin or cycloheximide), turnover of the transaminase virtually ceased. Isotopic labeling experiments, followed by immunological isolation of the enzyme, revealed that the failure of the enzyme level to change (after these inhibitors were given) reflected a stabilization of enzyme; that is, the enzyme level stayed constant because it was undergoing neither synthesis nor degradation under these conditions. This result raised the possibility that turnover of this enzyme is linked to its synthesis, which in turn suggests the intriguing idea that in some instances the ribosomal machinery for protein synthesis can "run backward," effecting both the synthesis and the degradation of a protein. Experiments in which RNA synthesis was inhibited by actinomycin have clearly excluded this possibility. In some experiments with this drug, transaminase synthesis was almost completely blocked, but the turnover process continued at the usual rate. Thus it is clear that the two processes of synthesis and degradation are not linked in an obligatory fashion. In other experiments both synthesis and degradation were blocked by actinomycin, and in still others neither of these parameters was affected to a significant extent. The reasons for these variable responses have not yet been clearly identified, but preliminary indications are that vigorous protein synthesis (and not RNA synthesis) is required for normal turnover. If this relationship holds, it may indicate that cessation of protein synthesis allows accumulation of some intermediate which stabilizes the transaminase against degradation.

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26.41 REGULATION OF ENZYME SYNTHESIS AND CHANGES IN TRANSFER RNA'S DURING DIETHYLNITROSAMINE CARCINOGENESIS

F. T. Kenney Joseph Kendrick
Lawrence Rosen L. E. Roberson

Diethylnitrosamine (DEN) is a potent hepatic carcinogen. Much experimental evidence supports the view that its carcinogenicity is related to its capacity to alkylate nucleic acid bases, yielding ethylated derivatives which render the nucleic acids abnormal or nonfunctional. The precise role of the various species of nucleic acids in hormonal regulation of enzyme synthesis is not yet known, but there is little doubt that properly functioning nucleic acids are required for hormonally induced enzyme synthesis. Induction of enzyme synthesis would thus appear to be a highly sensitive probe for abnormal nucleic acids and thereby for the earliest cellular changes during DEN carcinogenesis. On this premise we are examining the effects of DEN induction of hepatic tyrosine transaminase by hydrocortisone and by glucagon, each of which initiates a rapid increase in synthesis of this enzyme but by separate mechanisms. Pathogen-free male rats are being given DEN under conditions (5 mg kg^{-1} day^{-1}) in which the chronology of pathological changes at the cellular level has been documented. At intervals, groups of these rats are adrenalectomized and then tested for their response to the two hormones, along with appropriate controls. As this experiment is now only in its initial phase (35 days of DEN treatment), no significant variation from the normal response has yet been observed. We are hopeful that the changes which occur will yield significant information concerning the action of the carcinogen at the molecular level, and perhaps concerning the role of various RNA species in enzyme induction, as well.

Changes in transfer RNA's may be significant in enzyme induction. In other sections of this report (See Sect. 17) experiments are described which implicate ethylation of the bases of transfer RNA's as an integral part of the carcinogenic response to ethionine. Transfer RNA will be isolated from the livers of the rats being fed DEN, and analyzed to determine if the pattern of ethylation is similar for the two alkylating agents. This is expected to give insight into the pathway(s) by which alkyl groups are shifted from carcinogen to nucleic acids in vivo. It may also be possible to correlate tRNA changes with alterations in the induction response to hormones.

26.42 EARLY AND LATE EFFECTS OF HYDROCORTISONE ON THE SYNTHESIS OF TYROSINE TRANSAMINASE IN RAT LIVER

J. L. Wittliff F. T. Kenney

Garren and coworkers,[1] investigating the mechanism of regulation of enzyme synthesis by hydrocortisone, have suggested that induced enzyme synthesis stops about 7 hr after induction by hormone. Presently, we are measuring the rate of synthesis of tyrosine transaminase as a function of time after hydrocortisone, using radioimmunochemical techniques. In as little as 1 hr after hormone administration a fourfold increase in the rate of tyrosine transaminase synthesis is seen, whereas an increase in enzyme activity is not evident until 1.5 to 2 hr after induction. Three hours after induction the rate of synthesis was maximally increased. However, 9 hr after hormone administration (when the enzyme level is dropping) the rate of synthesis had returned to basal values rather than to zero. This result is contrary to the suggestion of Garren *et al.* that the rate of synthesis of induced enzyme is zero during the late phase of the induction curve, and fails to support the concept of a cytoplasmic (translational) repressor being responsible for the drop in enzyme level.

Reference

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26.43 INDUCTION OF GLUCOKINASE IN CULTURED REUBER H-35 HEPATOMA

J. R. Reel F. T. Kenney

During the past few years studies from several laboratories[1-4] have disclosed that rat liver con-

tains a glucose-ATP phosphotransferase (glucokinase) which differs from the previously described hexokinases in having both substrate specificity and low affinity for glucose. In addition, the hexokinases are constitutive, while glucokinase fluctuates with the physiological state of the animal. The activity of glucokinase falls to low levels in fasting and alloxan diabetes, and it appears to play an important physiological role in the control of hepatic glucose metabolism under conditions of high blood glucose. Glucokinase induction in fasted rats can be obtained by administration of either glucose or insulin. Whether both compounds are indeed primary inducers or whether the administration of glucose elevates the endogenous level of insulin, giving rise to induction indirectly, has been a difficult question to answer in the whole animal. Since glucokinase is known to be unique to the parenchymal liver cell, we are presently attempting to answer this question directly by using a cultured hepatoma (Reuber H-35), which was originally derived from this cell type.

Glucokinase and hexokinase were assayed by the glucose-6-phosphate dehydrogenase-dependent spectrophotometric method, which involves coupling of the glucose phosphorylation step with the glucose-6-phosphate dehydrogenase reaction and following the appearance of NADPH at 340 mμ in a recording spectrophotometer.[5] The 105,000 $\times$ *g* liver supernatant fraction from adult male rats fed ad libitum was found to have glucokinase and hexokinase specific activities of 0.017 unit and 0.028 per milligram of protein, respectively; these values were in agreement with the results of previous workers. In contrast, initial attempts to obtain valid estimates of glucokinase and hexokinase activity in hepatoma cell cultures have met with difficulties not previously encountered in rat liver. The 105,000 $\times$ *g* supernatant fraction from the hepatoma was found to contain an endogenous substrate(s) and/or enzyme which so readily reduced NADP that it masked the glucose-phosphorylating system and soon caused NADP to become rate-limiting in the reaction mixture. In order to remove any small-molecular-weight components which might be responsible for this interference, the 105,000 $\times$ *g* supernatant fraction was dialyzed overnight before assays were conducted. Again, as in previous experiments, there was a considerable reduction of NADP by some endogenous source. This result obtained whether glucose, ATP, or dithiothreitol was present or not in the incubation system. Since dialysis did not appreciably remove the source of interference, it is possible that an enzyme is present which catalyzes a reaction utilizing NADP. We are presently seeking ways to eliminate this factor so that we may continue with the original study, namely, to study the effect of insulin and glucose on glucokinase in hepatoma cell culture.

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26.44 REGULATION OF TYROSINE TRANSAMINASE SYNTHESIS BY HYDROCORTISONE IN HEPATOMA CELL CULTURES

J. R. Reel F. T. Kenney

Earlier studies[1-3] from our laboratory have shown that hydrocortisone induces tyrosine transaminase in cultures of the minimal deviation Reuber H-35 hepatoma. From this observation it was apparent that hepatoma cell cultures offered a convenient in vitro system to study hormonal control of enzyme synthesis without the masking influence of the in vivo environment. Consequently, a study was undertaken to investigate the mechanism by which hydrocortisone induces tyrosine transaminase in these cultures.

Addition of hydrocortisone (10^{-6} *M*) to monolayer or suspension cultures elicited a 6- to 14-fold increase in tyrosine transaminase levels, the response being maximal by 12 hr and persisting for as long as 40 hr. If induced cells were placed in fresh steroid-free medium, the enzyme rapidly declined toward basal levels ($t_{1/2}$ = 4 to 6 hr). A similar estimate of the half-life of the enzyme has also been obtained from the kinetics of induction. From the time taken to increase to one-half of the steady-state induced level, we have ob-

served an enzyme half-life of about 6 hr. Although hydrocortisone did not influence total RNA synthesis, actinomycin D blocked induction. This suggests that hydrocortisone induces the enzyme via a selective stimulation of RNA synthesis. To investigate possible translational control mechanisms, the effects of actinomycin D on preinduced cells have been studied. In preliminary experiments, using an isotopic-immunochemical procedure which involves labeling and selective immunochemical precipitation of the enzyme, tyrosine transaminase synthesis was increased 12-fold above basal levels in log- and stationary-phase monolayer cultures preinduced for 24 hr with hydrocortisone. A 6-hr actinomycin D (5 μg/ml) treatment of preinduced cultures lowered enzyme synthesis to nearly the basal rate, while in noninduced cultures this inhibitor depressed enzyme synthesis to 20% of the basal rate. Thus the half-life of the messenger RNA for tyrosine transaminase appears to be somewhat less than 6 hr in both induced and noninduced cultures. The discrepancy between the effect of actinomycin D on induced tyrosine transaminase synthesis and the "superinduction" observed under similar conditions by Thompson, Tomkins, and Curran[4] is currently under investigation.

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26.45 HORMONAL REGULATION OF MITOCHONDRIAL ENZYMES FROM HEPATOMA CELL CULTURES

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Many liver enzymes, primarily the dehydrogenases, can be altered by the administration of thyroid hormone.[1] The most dramatic response (induction) was given by the mitochondrial form of α-glycerophosphate dehydrogenase, which is cytochrome linked. The mitochondrial enzyme was increased as much as 20-fold over the basal level, while the activity of the soluble form of the enzyme remained unchanged after hormonal treatment. In view of recent findings that mitochondria possess a unique protein synthesis system, we investigated the effect of thyroid hormone on mitochondrial α-glycerophosphate dehydrogenase from monolayer cultures of rat hepatoma cells (Reuber H-35). Neither thyroxine (10^{-6} *M*) nor triiodothyronine (10^{-6} *M*) altered the level of the mitochondrial enzyme after 72 hr of treatment. The cultures were also treated with testosterone (10^{-6} *M*), estradiol (10^{-6} *M*), progesterone (10^{-6} *M*), hydrocortisone (10^{-6} *M*), glucagon (8 λ/ml), insulin (10 λ/ml), epinephrine (12 λ/ml), growth hormone (10 λ/ml), thyroid-stimulating hormone (10 λ/ml), and α-melanocyte-stimulating hormone (10 λ/ml). At these concentrations none of the hormones altered the level of mitochondrial α-glycerophosphate dehydrogenase after 72 hr of exposure.

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AEC-NIGMS	
Mammalian Comparative Mutagenesis (26.46–26.49)	**Biological Macromolecular Separations Technology (26.50–26.53)**
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26.46 DIFFERENTIAL INDUCTION OF DOMINANT LETHAL MUTATIONS IN MICE BY VARIOUS ALKYL METHANESULFONATES

U. H. Ehling D. G. Doherty H. V. Malling

From our study of the genetic effect of methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and a monofunctional nitrogen mustard derivate of acridine (ICR-170) we reached the conclusion that cross-linkage of the DNA strands may be an important molecular event in the induction of dominant lethals in mice.[1,2] In the further testing of the cross-linkage hypothesis we have extended the study to include two more esters of methanesulfonate: *n*-propyl methanesulfonate (n-PMS) and isopropyl methanesulfonate (iso-PMS). Elucidation of the general mutation mechanism of both compounds is in process in *Neurospora* (Malling, Ehling, Doherty, and de Serres). One aim of the comparative mutagenesis studies is to characterize, at the molecular level, the genetic mechanisms of induced dominant lethal mutations in mice. Another aspect of these studies is the exploration of the relation of chemical constitution and mutagenic activity.

Hybrid male mice (101 × C3H)F_1, 10 to 12 weeks old, were injected intraperitoneally with a single nonlethal dose of PMS, or with an equal volume of Hank's balanced salt solution. In the EMS experiment, two application methods were used: intraperitoneal and intravenous (tail vein) injection. Immediately after injection each male was caged separately with a (101 × C3H)F_1 or a (C3H × 101)F_1 female, 10 to 12 weeks old. A second mating was made two days after treatment. The males were then transferred to new females at four-day intervals.

Dominant lethal mutations cause early embryonic death of first-generation offspring. We therefore calculated the yield of induced dominant lethal mutation as

$$1 - \frac{\text{embryos exptl group/♀}}{\text{embryos control group/♀}}.$$

If the treatment is ineffective, this calculation should show no statistically significant deviation from zero.

Like MMS and EMS,[2] n-PMS induced a high frequency of dominant lethal mutations in spermatozoa and late spermatids and a low frequency in early spermatids. No mutations were induced in spermatocytes. The productivity of sperm treated as spermatogonia was not affected. In contrast, with iso-PMS, the spermatocyte stage was the most sensitive one for the induction of dominant lethal mutations. In addition, this chemical caused spermatogonial killing, which resulted in sterility a few weeks after treatment (Table 26.46.1).

Table 26.46.1. Frequency of Dominant Lethal Mutations (%)

Time of Matings (days post injection)	Compound					
	iso-PMS		n-PMS		EMS	
	50 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg	400 mg/kg	400 mg/kg
1–2	4.8	(Sterile)[a]	13.0	18.0	23.9	23.9
3–6	11.8	100.0	−6.6	37.9	56.3	70.8
7–10	13.9	(100.0)[b]	−5.0	78.3	(100.0)	(100.0)
11–14	9.7	84.7	15.6	45.2	84.8	(100.0)
15–18	7.6	85.0	5.5	31.8	40.3	31.3
19–22	−2.6	85.0	4.3	10.4	4.5	13.2
23–26	27.4	80.0	13.6	8.5	1.1	−8.6
27–30	48.7	88.6	−8.6	−4.4	7.7	7.7
31–34	85.9	Sterile	7.6	−2.0	−11.1	−25.0
35–38	Sterile	Sterile	8.7	c	−15.3	−13.5
39–42	Sterile	Sterile	−4.2		−14.5	18.5
43–46	Sterile	Sterile	0		5.8	7.6
Injection	ip	ip	ip	ip	iv	ip
No. of females in each mating interval	20	10	10	10	15[d]	15[d]

[a]Very likely due to a combination of two factors: reduced mating frequency and loss before implantation.
[b]This and the other figures enclosed in parentheses are very likely due to loss before implantation.
[c]Test incomplete.
[d]From 23 days post injection, only five females were used in each group.

The duration of the period of infertility depends on the injected amount of the compound. With 50 mg/kg of iso-PMS, the frequency of fertile matings begins to decline 23 days after injection. No fertile matings were observed 35 to 56 days after injection. The control level of fertile matings is reached about 78 days after treatment. The injection of 200 mg/kg of iso-PMS reduced the frequency of fertile females in all mating intervals and reached zero 31 days after injection. The males in this group have not recovered their fertility 170 days post injection.

A comparison of the yield of induced mutations of compounds with different reaction kinetics must take into consideration the possibility that the route of administration affects the mutation yield. We have therefore compared the effect of intravenous and intraperitoneal injection of 400 mg/kg of EMS. The yield of EMS-induced dominant lethal mutations is not appreciably, if at all, affected by the injection method (Table 26.46.1). We are now testing the effect of intravenous injection of 50 mg/kg of iso-PMS.

In equimolar dosages the relative effectiveness of the three alkyl methanesulfonate compounds that had similar qualitative actions in inducing dominant lethal mutations in spermatozoa and spermatids is in decreasing order as follows: MMS > EMS > n-PMS. The efficiency decreased by a factor of approximately 4. The toxicity of the compounds decreased in the same order by a factor of 1.5 to 2.0. Studies are now in progress to determine the biochemical mechanism that is responsible for the qualitatively different mutagenic actions of n- and iso-PMS.

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26.47 INDUCTION OF SPECIFIC LOCUS MUTATIONS BY ALKYLATING AGENTS IN MALE MICE

U. H. Ehling R. B. Cumming
H. V. Malling

The purpose of these studies is to elucidate the molecular mechanism of gene mutations at seven loci in mice. The design of the experiments to achieve this goal is based on the working hypothesis that it will be possible to characterize the gene mutations in mice by testing their mutability with compounds whose specific mutagenic action is well established. In phages, the base-pair transitions induced by methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) are preferentially from GC to AT.[1] In *Neurospora*, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induces mainly, or exclusively, base-pair transitions in both directions but predominantly from AT to GC,[2] whereas 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino]acridine dihydrochloride (ICR-170) induces mainly base-pair insertions and deletions.[3] The same compounds were also tested for the induction of dominant lethal mutations in mice.[4,5]

Hybrid male mice, (101 × C3H)F_1, 10 to 12 weeks old, were injected intraperitoneally with a single nonlethal dose of the test compound, or with an equal volume (1 cm^3) of Hank's balanced salt solution. Immediately following injection, each male was caged separately with a T-stock female, 10 to 12 weeks old. The males were transferred to a new T-stock female eight weeks after the treatment. Two hundred seventy treated males were first tested for the induction of dominant lethal mutations[4] and later mated to T-stock females. The method for scoring the specific locus mutations has been described by W. L. Russell.[6]

Table 26.47.1. Frequency of Specific Locus Mutations Induced by Alkylating Agents

Germ Cell Stage Tested	Agent	Dose (mg/kg)	Number of F_1 Offspring	Number of Mutations at 7 Loci
Postspermatogonial	MMS	150	558	1[a]
	EMS	250	724	0
		400	511	0
	MNNG	50	894	0
	ICR-170	4	1092	0
Spermatogonial	MMS	50	555	1[b]
		100	751	1
		150	2660	0
	EMS	100	432	0
		200	779	0
		250	2442	0
		400	1579	0
	MNNG	50	3472	0
	ICR-170	4	3084	0
Control group	Hank's balanced salt solution		4691	0

[a]Died before weaning.

[b]Test incomplete.

The results of the specific locus experiments are summarized in Table 26.47.1. Until now no specific locus mutations have been found after treatment of males with EMS, MNNG, and ICR-170. MMS induced mutations at the piebald spotting (*s*) locus and albino (c^a) locus. The *s* mutant was induced in early spermatids about 19 days after injection of the male with 150 mg/kg of MMS. The mutant died when it was 12 days old. The treated male produced, until now, a total of 45 offspring. One albino locus mutant, a male, was conceived about 224 days after treatment of the male with 100 mg/kg of MMS. The other albino locus mutant (probably to the chinchilla allele), a female, was conceived about 331 days after treatment of the male with 50 mg/kg of MMS. The first albino locus mutant was produced by a male with a total of 45 offspring; the second mutant at this locus comes from a male with 60 offspring (Table 26.47.1). One spotted presumed mutant has occurred in the MNNG experiment. The mutant is too young for a genetic test, but the phenotype is not typical for the *s* locus and resembles some dominant spotting mutations.

The data suggest the possibility that moderate doses of MMS may be more effective in inducing specific locus mutations than a dose of 150 mg/kg. This should be explored further, because a phenomenon of this type has been found for x-ray doses.[7]

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26.48 CHEMICAL INDUCTION OF DOMINANT LETHALS IN FEMALE MICE

W. M. Generoso

In female mammals, reduction in the number of live embryos following prefertilization treatment with chemical mutagens may be due to genetic damage or to the interference of the mutagen with the normal oogenesis, ovulation, fertilization, or maternal physiology. Such factors that make dominant lethal induction studies in adult female mammals difficult have been dealt with in detail.[1] Mainly because of these complications, very few studies have been made on the chemical induction of dominant lethals in females. Proof of genetic nature of chemical effects on the female germ cells has, therefore, been wanting. It is obvious that reduction in either litter size or number of live embryos, alone, as a result of prefertilization treatment of females, is not an adequate criterion for evaluating the genetic effects of an alkylating agent. In this study, direct evidences of true genetic effects of a chemical compound on the germ cells of the female mouse are presented.

T-stock females, 11 to 13 weeks old, were injected intraperitoneally with a sublethal dose of one of the following: 325 mg/kg ethyl methanesulfonate (EMS), 150 mg/kg methyl methanesulfonate (MMS), 70 mg/kg *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), or 4 mg/kg 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino] acridine dihydrochloride (ICR-170). The sublethal doses, determined in the preliminary tests, caused few deaths. In this laboratory, EMS and MMS were found effective in the induction of dominant lethal mutations in male (101 × C3H)F_1 hybrid mice, while MNNG and ICR-170 were found ineffective.[1] The controls received an equal volume (1 ml) of Hank's balanced salt solution. Batches of identically treated females were caged individually with (101 × C3H)F_1 males at intervals 0, 5, 10, and 15 days after treatment. The males were separated from the females after the vaginal plugs (indication of mating) were observed. Females were then opened 14 to 17 days after mating and scored for the number of corpora lutea, live embryos, and deciduomata. The frequency of dominant lethals was calculated as

$$1 - \frac{\dfrac{\text{living embryos}}{\text{corpora lutea}} \text{ (for experimental females)}}{\dfrac{\text{living embryos}}{\text{corpora lutea}} \text{ (for control females)}} .$$

The results are summarized in Table 26.48.1. Because the mean numbers of corpora lutea per female were not significantly different between the treatments, the mean numbers of live embryos

Table 26.48.1. Induction of Dominant Lethals with Chemicals

Compound	Dose (mg/kg body wt)	Treatment to Fertilization Interval (days)	Number of Females	Fertile Matings (%)	Average Number of Corpora Lutea[a]	Average Number of Implants[a]	Average Number of Live Embryos[a]	Induced Dominant Lethals (%)
HBSS	1 ml	½–4½	84	94.0	10.6	9.3	7.8	0
(control)		5½–9½	75	90.7	10.3	8.3	6.6	0
		10½–14½	66	90.9	10.9	8.8	7.2	0
		15½–19½	54	90.7	10.6	8.4	6.8	0
EMS	325	½–4½	40	20.0[b]	10.4	4.8	1.5[b]	80.35
		5½–9½	76	67.1[b]	10.6	5.6	3.3[b]	52.23
		10½–14½	26	88.5	11.4	7.6	5.9[c]	21.58
		15½–19½	28	96.4	11.0	8.3	6.3	10.09
MMS	150	½–4½	62	33.9	9.9	4.2	2.2[b]	69.94
		5½–9½	53	62.3[b]	10.9	6.9	5.3[b]	24.29
		10½–14½	39	82.1	10.7	7.9	6.3	10.00
		15½–19½	21	100.0	11.1	8.5	7.2	−1.87
MNNG	70	½–4½	38	86.8	10.7	7.9	6.0[b]	24.36
		5½–9½	32	90.6	11.3	8.7	7.4	−1.33
		10½–14½	31	90.3	10.6	9.0	7.0	−0.06
		15½–19½	34	91.2	11.1	8.9	7.3	−2.14
ICR-170	4	½–4½	36	94.4	11.1	9.4	7.8	4.10
		5½–9½	32	90.6	10.5	8.2	6.8	−0.82
		10½–14½	32	78.1	11.5	9.6	7.7	−1.61
		15½–19½	30	80.0	11.1	8.9	7.4	−3.91

[a]Per fertile female.
[b]$P < 0.01$.
[c]$P < 0.05$.

for the control and chemicals were compared directly. Significant reductions in the average number of live embryos per female were found in the EMS, MMS, and MNNG lots. The effect of EMS was maximum during the first 4½ days after treatment, gradually decreasing up to 14½ days, after which time no significant reduction in the number of live embryos was observed. A similar trend was found with MMS, but the statistically significant effects were limited to within 9½ days after treatment. The effect of MNNG was significant only during the first 4½ days.

In the EMS and MMS experiments the frequencies of dominant lethals and the reduction in average numbers of live embryos may be gross underestimations of the total genetic effects, since they were calculated from fertile matings only. All females that had implants were considered fertile. In both EMS and MMS there was a high incidence of sterility among females mated within 6½ days after injection. Females treated with MNNG and ICR-170 had normal frequency of fertile matings throughout the ½ to 19½ days' test period. The females treated with chemicals had normal frequency of matings.

The high frequency of sterile matings, in addition to the marked reduction in the numbers of total implants and live embryos, in the EMS experiment (½ to 4½ days) provided an excellent situation for studying whether or not the mechanism of chemical effects was through genetic damage. The effects of EMS on the rates of ovulation and fertilization and preimplantation embryonic development were, therefore, studied among females mated during the first 4½ days after treatment. Phase-contrast microscopy of

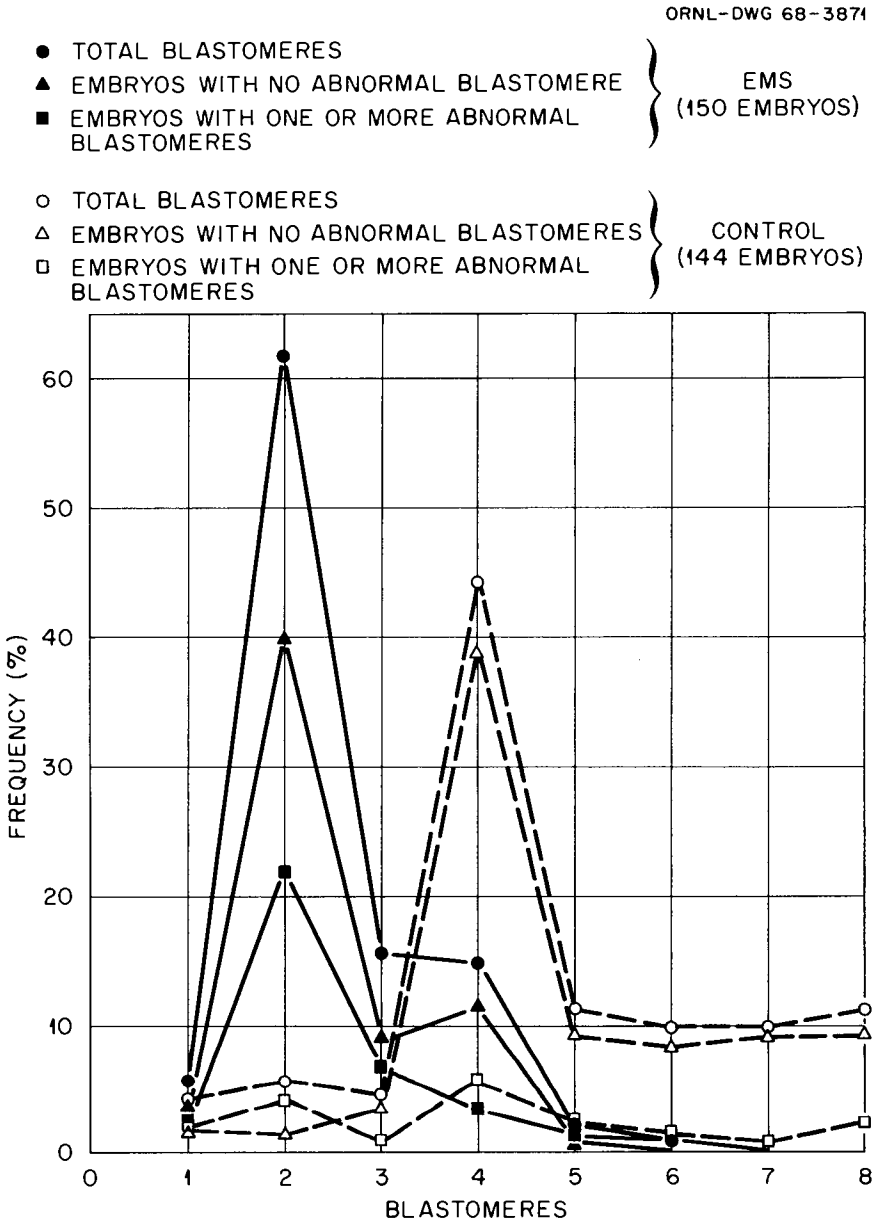

Fig. 26.48.1. Distribution of Embryos Fixed Approximately 48 hr After Fertilization and Scored According to the Total Number of Blastomeres and Presence or Absence of One or More Abnormal Cells.

the tubal eggs showed that the mated females had ovulated and had a normal rate of fertilization. There was, however, an indication that EMS treatment increased the number of ovulated eggs. This increase was not detected when the corpora lutea were counted, because of the difficulty in identifying a corpus luteum when the number of developing embryos is small. Additional evidence of EMS-induced superovulation was found in the materials fixed approximately 24 hr after sperm entry, in which the EMS-treated females had significantly more embryos than the controls. This result is similar to that of x ray.

Serial sectioning of oviducts fixed approximately 24 hr after sperm entry revealed the normal development of embryos in the EMS-treated females. At this time 92.8% (165 out of 178) of the embryos in the EMS lot were in the two-cell stage. In the control, 84.9% (129 out of 152) of the embryos had two blastomeres. A very important finding, however, was the marked increase in the frequency of embryos with subnuclei. In the EMS lot, 15.2% (27 out of 178) of the embryos had subnuclei, compared with 1.3% (2 out of 152) in the control. The presence of subnuclei is here taken as a direct evidence of chromosomal breakage.

The progress of embryonic development in the EMS and control lots was further compared by examining the embryos fixed about 48 hr after sperm entry. The embryos were scored for the total number of blastomeres and presence or absence of abnormal cells. The presence of a true nucleus and absence of a subnucleus was considered normal. The results are shown in Fig. 26.48.1. Treatment with EMS had checked the development of the majority of the embryos at the two-cell stage. These embryos were probably incapable of further development and, together with the high incidence of embryos with one or more abnormal blastomeres, provide additional evidence for the genetic nature of EMS effect.

Comparison based on the sublethal dose showed that EMS was more effective than MMS and MNNG, in this order, in the induction of dominant lethals. Sterility due to EMS treatment, and for practical purposes also of MMS, was attributable to the induced dominant lethals, in which the genetic effects were already evident as early as the two-cell embryonic stage. Cytological examination of the genetic effect of MNNG was not attempted because of the difficulty of histological investigation when the frequency of dominant lethal mutations is low. No induction of dominant lethals was observed with ICR-170.

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26.49 STRAIN VARIATION IN THE SENSITIVITY OF MICE TO ALKYLATING AGENTS

W. M. Generoso

Strains differ in many physiologic characteristics as a result of their differences in the frequency of allelic genes at a great number of loci. It is the purpose of this experiment to determine the effect of the genotype on the mutagenic action of a variety of alkylating compounds of known molecular structure.

Eleven- to thirteen-week-old females of the T-stock (random bred), (101 × C3H)F_1, and (SEC × C57BL)F_1 strains were injected intraperitoneally with 325 mg/kg ethyl methanesulfonate, or 150 mg/kg methyl methanesulfonate, or 1 ml of Hank's balanced salt solution. We found in the previous experiment with female mice that the stage of oocyte development in the last few days prior to ovulation is most sensitive for dominant lethal induction with ethyl methanesulfonate (EMS) or methyl methanesulfonate (MMS). Preliminary results also showed that there was an abnormally high frequency (about 50%) of females that mated within the first ½ day after EMS or MMS injection in the (SEC × C57BL)F_1 strain. These females were largely sterile, probably not due to dominant lethal mutations, since the fertile matings during 1½ to 5½ days after treatment yielded very low or no induced dominant lethal mutations, but probably because they accepted the males at the wrong stage of the oestrus cycle. This complication will be investigated further. Comparison between strains was, therefore, based on fertilization within 1½ to 5½ days after treatment.

Results shown in Table 26.49.1 revealed marked strain variability in dominant lethal induction with

Table 26.49.1. Strain Variation in Dominant Lethal Induction with EMS and MMS in Matings Occurring from 1½ to 5½ Days After Treatment

Strain	Compound	Number of Females	Fertile Matings (%)	Average Number of Corpora Lutea[a]	Average Number of Implants[a]	Average Number of Live Embryos[a]	Induced Dominant Lethals (%)
T stock	Control[b]	77	92.2	10.5	9.1	7.5	0
	EMS[c]	56	28.6	10.0	4.0	1.6	77.36
	MMS[d]	71	32.4	10.0	4.1	2.2	68.97
(101 × C3H)F_1	Control	27	96.3	7.4	6.8	6.2	0
	EMS	22	90.9	9.3	7.5	6.3	19.71
	MMS	21	90.5	8.4	7.0	5.6	20.99
(SEC × C57BL)F_1	Control	30	76.7	10.3	9.3	9.1	0
	EMS	22	81.8	10.8	9.4	8.8	7.59
	MMS	25	72.0	11.3	9.3	7.66	22.96

[a]Per fertile female.

[b]All controls received 1 ml of Hank's balanced salt solution.

[c]All EMS doses were 325 mg per kilogram body weight.

[d]All MMS doses were 150 mg per kilogram body weight.

chemicals. T-stock females were much more sensitive to dominant lethal induction with EMS or MMS than the hybrids. Treatment with EMS did not reduce the number of live embryos in either hybrid. However, there was a slight increase in the frequency of dominant lethal mutations induced with EMS in the (101 × C3H)F_1 females. In this strain, EMS caused an increase in the number of eggs ovulated, as shown by the corpora lutea counts, which were lost during the pre- and post-implantation stages. In the (SEC × C57BL)F_1 EMS-treated females, the very low frequency observed probably represents very little or no induction of dominant lethals. MMS caused a slight reduction in the average number of live embryos and also induced a slight increase in the frequency of dominant lethal mutations in the hybrid females. These findings, the repeatability of which will be checked with identical experiments, showed that on the sublethal dose basis, EMS was more effective than MMS in the T stock, MMS more effective than EMS in the (SEC × C57BL)F_1, while in the (101 × C3H)F_1, EMS and MMS had similar effectiveness.

The average numbers of live embryos and the frequencies of induced dominant lethal mutations were calculated from fertile matings only. Comparisons on this basis probably underestimate the true differences between strains. In the T stock, EMS and MMS caused a marked reduction in the frequency of fertile matings. Normal frequency of fertile matings was found among the experimental females of both hybrids. We have found that the EMS-induced sterility in the T-stock females resulted from the inability of the embryos to develop normally due to dominant lethal mutations.

It is clear that one strain may reveal a strong mutagenic action of a specific compound in the females while another may not. We do not yet know the reasons for this, but the physiologic uniqueness of a strain may manifest itself in one or more ways, such as differential enzymatic degradation of the chemical, which may result in different concentrations of the active compound reaching the germ cells. We are presently testing in females the response of the above strains to dominant lethal induction with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino] acridine dihydrochloride (ICR-170).

In this laboratory, EMS and MMS at the nonlethal doses of 200 and 50 mg per kilogram body weight,

respectively, were shown to cause an increase in the frequency of dominant lethal mutations of about 40% in the late spermatids (conception 8 to 14 days after treatment) of (101 × C3H)F_1 hybrid males.[1] In the females of this strain the sublethal doses of 325 mg/kg EMS or 150 mg/kg MMS induced only about 20% dominant lethal mutations. We now have an experiment designed to find out if this striking sex difference in sensitivity to dominant lethal induction with EMS is also true in the T-stock and the (SEC × C57BL)F_1 strains.

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26.50 CHANGES IN *E. COLI* LEUCINE tRNA AFTER BACTERIOPHAGE T2 INFECTION

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The chromatographic profile of leucine tRNA from *E. coli* is changed markedly following infection with bacteriophage T2. Specifically, there appears to be a quantitative change in those species normally present in uninfected cells, which occurs early in infection. In addition, one or possibly two new peaks are detected at about 20 min post infection.[1] These chromatographic changes we refer to as "early" and "late" changes respectively. Our immediate efforts have been directed toward determining the cause or causes of these changes.

Two possible causes for the changes are being considered, modification of existing species and/or de novo synthesis. Sueoka and Sueoka[2] have proposed the former as best explaining the "early" change. Their proposal is based on: (1) their finding that the total acceptor activity for leucine relative to several other amino acids did not change after infection and (2) studies using yeast synthetases. We have preliminary data which are inconsistent with their explanation. In fact – in our hands – leucine acceptor activity has decreased significantly in 6' T2 tRNA when compared with normal. Moreover, this decrease can be demonstrated chromatographically and appears to be reflected in only one of the four (or possibly five) peaks of normal leucyl-tRNA resolvable by Freon chromatography.[3] Our data are best explained by an apparently specific inactivation of one of the leucine isoaccepting species found in normal cells. In view of the discrepancy with the Princeton group, these experiments are being repeated and expanded.

Because the "late" change is a qualitative one with the new peaks being completely separable from any of the leucine tRNA's found in normal cells, we feel that it is more amenable to further experiment. Accordingly, labeling experiments designed to detect possible de novo synthesis have been done. To date, labeling studies with ^{3}H- or ^{14}C-uracil or ^{32}P have yielded ambiguous results. This, we feel, is possibly due to our inability to rid our tRNA preps of all other types of nucleic acids. Experiments using ^{35}S-labeled sulfate are consistent with an origin by de novo synthesis of the new peaks (tRNA has been shown to be the only major class of nucleic acids which contains thiolated bases). The results are not clear-cut, however. Although there is a region (on Freon chromatography) of relatively high specific activity which coincides with the new leucine peaks, there is a considerable amount of labeling of the other tRNA's (about one-fourth to one-fifth of the specific activity of a similarly labeled preparation from normal cells) and also there is sulfur-containing material which elutes from Freon after the new leucine peaks. At present we have no evidence for any amino-acid-accepting activity in this region of the chromatogram. In general our studies with ^{35}S are in agreement with those reported by Hsu, Foft, and Weiss[4] concerning differences in the labeling pattern before and after injection.

Chromatography of the ^{35}S-labeled preparation on the isoamyl acetate column[5] resulted in a loss of sulfur, which was not reflected in either RNA (optical density) or ^{3}H-leucine recovery. β-Mercaptoethanol in the eluting buffer prevented this loss. This might prove a good tool for determining the biological role for the thiolated bases in tRNA. One approach toward the question of de novo synthesis or modification was to look for a difference in the amino-acid-carrying terminal polynucleotide sequences produced by T_1 RNAase. No difference was detected between normal and 2-hr infected tRNA. At least the terminal sequences in the new peaks are not grossly different from those in normal leucine tRNA.

Perhaps DNA-RNA annealing studies will help to conclusively ascertain the origin of the new peaks. Preliminary experiments indicate that tRNA from infected cells will anneal to T2 DNA.

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26.51 ISOLATION OF TWO FORMYLMETHIONINE TRANSFER RNA's FROM *ESCHERICHIA COLI* BY REVERSED-PHASE CHROMATOGRAPHY

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Following the discovery of formylmethionyl-tRNA,[2] its role as a chain initiator in *E. coli* protein synthesis was established.[3,4] The codons for $tRNA_F^{Met}$ have been shown to be AUG, GUG, and UUG.[4–6] It was pointed out[6,7] that the recognition of AUG and GUG by the same tRNA was not a permissible multiple codon recognition by the Crick wobble hypothesis,[8] and the possible presence of two $tRNA_F^{Met}$ species was postulated.

E. coli B crude tRNA was fractionated by reversed-phase chromatography using a system composed of 5% trioctylpropylammonium bromide on Chromosorb W. Three methionine tRNA's were separated by this system. Two of the $tRNA^{Met}$ peaks gave equivalent values for methionine acceptance and formylation. The first formyl-accepting species ($tRNA_{F1}^{Met}$) was found to be free of other tRNA's, while the second species ($tRNA_{F2}^{Met}$) was found to contain valine and lysine tRNA's. Rechromatography of the $tRNA_{F2}^{Met}$ material resolved these tRNA's. A mixture of (^{14}C) Met-$tRNA_{F1}$ and (^{3}H) Met-$tRNA_{F2}$ was separated by rechromatography. Both formyl-accepting species were found to bind to ribosomes under the direction of poly AUG and UG; however, this response was affected differently by the Mg^{2+} concentration.

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26.52 RESTORATION OF AMINOACYLATION ACTIVITY OF UNDERMETHYLATED tRNA BY IN VITRO METHYLATION

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Little if any difference in the amino-acid-acceptance activities of normal (fully methylated) and undermethylated tRNA preparations from *Escherichia coli* have been observed.[1] The results of Peterkofsky *et al.*,[2] however, indicated that normal tRNA may have a somewhat higher capacity to accept amino acids than undermethylated tRNA. Limitations on the interpretation of these results have been pointed out.[1,3]

In a mixture of undermethylated tRNA's from *E. coli*, we have found that for four amino acids tested (Phe, Leu, Tyr, and His), the corresponding tRNA's showed decreased aminoacylation activities when compared with normal tRNA from the same organism. Upon in vitro methylation of the undermethylated tRNA's with methylases free of RNase, a change in the aminoacylation activities occurred. With two tRNA species (Phe and His), complete restoration of aminoacylation activity was observed. The activity of $tRNA^{Leu}$ was partially restored, and $tRNA^{Tyr}$ was refractive to restoration. The distribution of in-vitro-incorpo-

rated methyl groups in the mixture of undermethylated tRNA's, analyzed by thin-layer chromatography, showed that approximately 90% of the methyl groups were incorporated as a methylated uridine phosphate.

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26.53 STUDIES ON THE PHENYLALANINE tRNA SYNTHETASES OF *NEUROSPORA CRASSA*. PURIFICATION

F. J. Kull K. Bruce Jacobson

Neurospora crassa contains both cytoplasmic and mitochondrial phenylalanine tRNA synthetases. The mitochondrial enzyme is able to utilize either $tRNA^{Phe}$ of *N. crassa* mitochondria or $tRNA^{Phe}$ of *Escherichia coli* for esterification of phenylalanine.[1] The cytoplasmic synthetase, however, has the unique property of being able to aminoacylate both $tRNA^{Ala}_{E.coli}$ and $tRNA^{Val}_{E.coli}$ as well as $tRNA^{Phe}_{N.crassa}$.[2,3] Attention has been directed toward purification and isolation of this enzyme as a necessary prerequisite to projected studies concerning the mechanism of tRNA aminoacylation using highly purified tRNA preparations. The concomitant purification of the valine tRNA synthetase of *E. coli* will allow comparison of the activities of these enzymes with respect to $tRNA^{Val}$ of *E. coli*.[4] Chemical modification of tRNA and kinetics and various physical parameters of enzymes, tRNA's, and their complexes when combined may aid in better understanding the mechanism of tRNA aminoacylation.

The purification procedure employed for isolation of the cytoplasmic phenylalanine tRNA synthetase of *N. crassa* includes: a 75% ammonium sulfate precipitation of a postmitochondrial supernatant fraction, a 2.0 *M* ammonium sulfate back extraction of this precipitate, a batch hydroxylapatite elution step at pH 7.0, a DEAE-Cl cellulose column chromatography step, and hydroxylapatite column chromatography at pH 7.9. The last step separates the enzymic activity (assayed using a mixture of $tRNA^{Val}_{E.coli}$ and $tRNA^{Ala}_{E.coli}$ and ^{14}C-phenylalanine) into two fractions. The first activity eluting from the column (A enzyme) has been purified about 60-fold. The second active fraction (B enzyme) has been purified with an increase in specific activity of about 1000 over the starting material. This enzyme fraction appears to be in a state approaching homogeneity as evidenced by: (1) constant specific activity across a symmetrical optical density and activity peak upon rechromatography on hydroxylapatite and (2) electrophoresis at pH 8.5 which indicates a single protein staining band. The two activities appear to be separate enzymes, as they can be rechromatogrammed separately without redistribution of activity. This essentially rules out an equilibrium between two forms of the same enzyme. The two activities do, however, exhibit similar behavior toward $tRNA^{Val}_{E.coli}$, $tRNA^{Ala}_{E.coli}$, $tRNA^{Phe}_{E.coli}$, and unfractionated *N. crassa* cytoplasmic tRNA. Experiments are in progress to further establish the character of these two activities.

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AEC-NASA SPACE BIOLOGY

Space Biology – High-Energy Proton Studies (26.54, 26.55)

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26.54 ACUTE EFFECTS OF 62-Mev PROTONS IN MICE

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The following results were obtained as part of a study of the relative biological effectiveness (RBE) of 62-Mev protons in mice.

Approximately 2000 RF/Un female mice were exposed to graded doses of either 300-kv x rays or 62-Mev protons given as a single exposure. Of the animals exposed, 528 x-irradiated and 436 proton-irradiated animals fell into the 1 to 99% mortality range, from which $LD_{50/30}$ values of 559.5 ± 7.6 rads (x rays) and 622.1 ± 9.4 rads (protons) were obtained. The RBE of 62-Mev protons was thus found to be 0.90 compared with 300-kv x rays. Mean survival times of groups exposed to either x rays or protons in the 1 to 99% lethal range were approximately 12 days (minimum 9 days), with no indication of the early death pattern that has been reported for higher-energy

proton exposures. At doses of 900 rads or more of either x rays or protons, however, the mean survival time was five days or less. A dose reduction factor of 2 was found when AET was injected prior to either proton or x-ray exposures. Postirradiation weight loss of the thymus, spleen, and testes also produced RBE's of 1 or less for 62-Mev protons compared with 300-kv x rays (Table 26.54.1).

Table 26.54.1. Relative Biological Effectiveness of 62-Mev Protons as Compared with X Rays for Acute Effects in Mice

End Point	ED_{50} (rads)		RBE
	X Ray	Protons	
Lethality (30 days)	560	622	0.9
Lethality (30 days) $\bar{c}$ AET	1150	1200	0.96
Thymus weight loss (2 days)	150	150	1.0
Thymus weight loss (5 days)	225	250	0.9
Spleen weight loss (2 days)	250	260	0.96
Spleen weight loss (5 days)	350	250	1.4
Testes weight loss (30 days)	325	400	0.81

It is concluded from these experiments that 62-Mev protons are no more effective and perhaps slightly less effective per rad than 300-kv x rays in producing thymus, spleen, and testes weight loss and mortality in the mouse.

Reference

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26.55 FURTHER OBSERVATIONS ON THE RBE OF 60-Mev PROTONS FOR LATE SOMATIC EFFECTS IN MICE

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To study the RBE of 60-Mev protons for late samatic effects in mammals, ~1600 eight-week-old RF female mice, including sham-irradiated controls, were exposed in replicates to 0–400 rads of whole-body 60-Mev proton radiation, as described previously.[2] Comparable groups of animals received similar doses of 300-kvp x rays. Preliminary findings, reported previously,[3] suggested the two radiations were equally effective for impairment of body weight and for induction of lens opacities in 30-day survivors through 150 days post exposure. Although a considerable number of the mice are still alive, further data on long-term effects have become available and are hence reported at this time.

To obtain interim information on life-shortening as a function of dose and type of radiation, the percentage of mice surviving in each of the replicates within a dose group has been analyzed as a function of age, and the age corresponding to 50% survival has been used to estimate the mean survival time for the respective dose group. The results indicate dose-dependent life-shortening following both types of radiations, with an RBE for all five dose groups between 1.0 and 1.2. Observations on lens opacification, based on approximately 3500 lens examinations on 529 animals, confirm and extend the earlier findings up to a year after irradiation.

These preliminary results on life-shortening and cataract induction are consistent with those on acute effects[4] in suggesting an RBE equal to, or slightly less than, 1.0 for 60-Mev protons as compared with 300-kvp x rays. This conclusion should be regarded as tentative, however, pending further observation and analysis of the data.

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26.56 MUTATIONAL RESPONSE OF *HABROBRACON* IN THE BIOSATELLITE II EXPERIMENT

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Introduction. – The Biosatellite II was launched at 1804 EDT on September 7, 1967. The spacecraft was successfully recovered at 1508 EDT on September 9, 1967, after 30 orbits rather than after 46 orbits as originally planned. The experiment was as well controlled as possible in that the temperature profile in the ground-based control setup closely followed the temperature profile of the spacecraft and in that the radiation sources in the spacecraft and the ground-based control setup were nearly identical in strength. Also, all postflight operations on the ground-based control animals at Cape Kennedy were carried out within an hour of the postflight operations on the flight animals at Hickam Field.

The *Habrobracon* part of the experiment was designed to scan a wide spectrum of mutations and other responses to radiation of adult males and females.

Results. – The first result noted was that every animal survived. The second result was that males that had been in orbit were disoriented in their mating behavior for two days after their flight.

Sperm. – The exposure-action curve for hatchability of fertilized eggs following irradiation of sperm in the flight vehicle and ground-based control setup is shown in Fig. 26.56.1. It can be seen that there is no significant difference between the flight and control animals. The differences among modules are small, verifying the essential similarity of replicate modules at each dose. Neither a synergistic nor an antagonistic effect of flight dynamics with radiation exists when sperm dominant lethality is the criterion.

The spontaneous recessive lethal mutation frequencies in sperm were the same in the spacecraft and the ground-based control setup. The recessive lethal mutation frequencies and frequencies of translocations induced in sperm by the ^{85}Sr γ radiation are still under analysis.

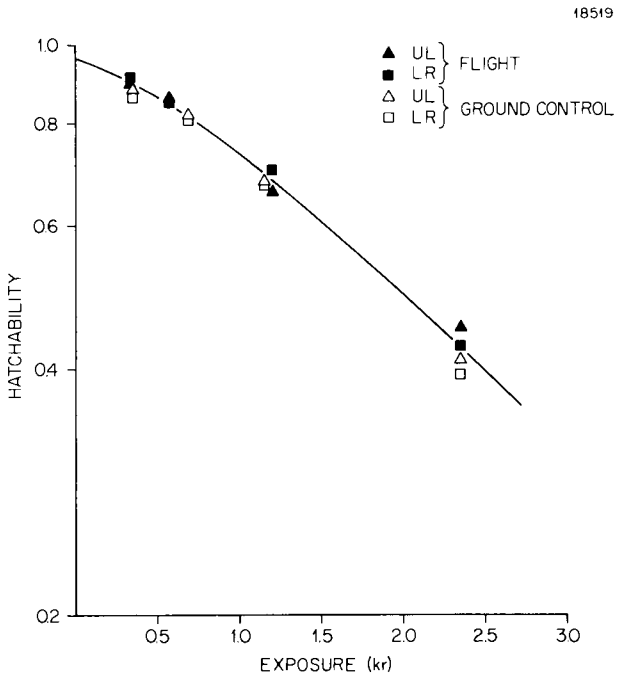

Fig. 26.56.1. The Hatchability of Fertilized Eggs from Females Mated to Irradiated Males.

Metaphase I Oocytes. – Exposure-hatchability curves of eggs irradiated during the first meiotic metaphase in the Biosatellite II experiment are shown in Fig. 26.56.2. A marked sensitivity difference exists between those irradiated in the spacecraft and on the ground. Those which had been orbited differed from module to module. It appears that a synergistic effect of flight dynamics with gamma radiation occurs when total lethality (dominant and recessive) in metaphase I oocytes is the criterion.

A similar difference exists between hatchabilities of metaphase I oocytes irradiated with an exposure of 2000 r prior to flight. However, the eggs from these females had higher hatchabilities than any of those irradiated during the flight. Recovery from the radiation damage to these eggs (and to those under irradiation) must have been occurring during the flight.

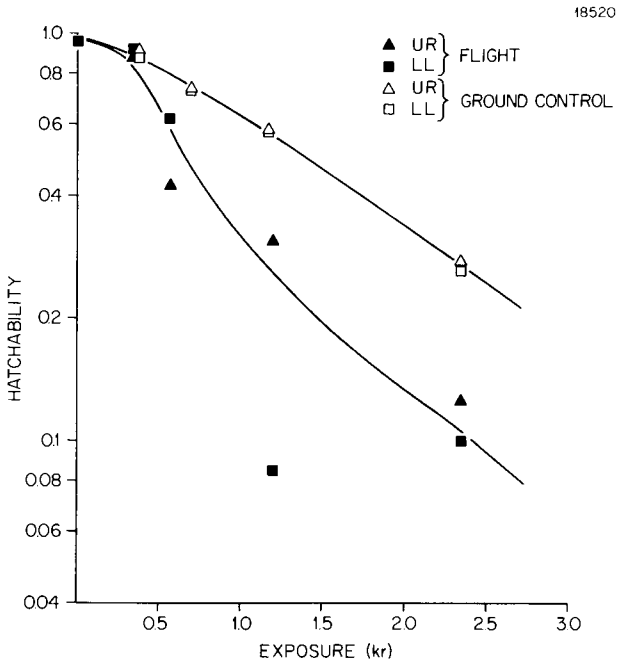

Fig. 26.56.2. The Hatchability of Unfertilized Eggs from Virgin Females. The eggs represent the class that were in the first meiotic metaphase at the time of irradiation.

Prophase I Oocytes. – At the exposures used, hatchability was above 90%; thus the only measurable parameter available to us was genetic recombination on chromosome I. Since genetic recombination is not affected by irradiation during prophase I, no differences were anticipated. None were observed.

Transitional and Primitive Oogonia. – Reproductive performance of the females was the principal criterion for effects on these stages; it depends upon efficiency of somatic tissues and requires normality in the processes of mitosis, meiosis, and reproduction. This part of the experiment was the principal responsibility of the group from the University of North Carolina.

The pattern of egg production for the *Habrobracon* females recovered from the spacecraft differed completely from results obtained with females that had been in the ground-based control setup. Normally, after the first three days a group of irradiated females will begin to produce fewer eggs than an unirradiated group. The irradiated group will go through a low period from five (acute irradiation) to eight or nine (protracted irradiation) days after being treated. The egg production then goes up but never to the level of the control.

The ground-based control females from the Biosatellite II experiment behaved as expected, but those from the spacecraft showed a compensatory pattern of egg production. Although egg production was slightly lower for irradiated flight females than the ground and flight controls for the first week, the egg production was higher than the 0-dose controls for the remaining portion of the 20-day study.

The data for females irradiated in the nominal 4000-r position (receiving approximately 2350 r in the shortened experiment) are shown graphically in Fig. 26.56.3. There is an antagonistic effect of flight dynamics with radiation in oogonia. The radiation response of these mitotically reproducing cells is effectively eliminated by the flight.

Life-Span. – The females carried in the spacecraft lived significantly longer than their control counterparts.

Discussion. – Null, synergistic, and antagonistic effects of space-flight factors with radiation were found in the *Habrobracon* experiment. We draw these conclusions from data obtained from replicate samples in slightly different geometric configurations within the flight vehicle and in the ground-based control setup. These are preliminary conclusions, since the final genetic analyses are far from complete.

Nevertheless, the results to date indicate that the null effects of space flight with radiation found with mature sperm are related to static, fully differentiated cells in which radiation-repair systems are minimally effective even under normal conditions. The synergistic effects of space flight with radiation appear to be associated with meiotic cells which are static but in which repair systems are fully effective. The antagonistic effects of space flight with radiation appear to be associated with mitotic cells, or with those cells transitional between mitosis and meiosis but in which repair systems are fully effective.

Three general hypotheses come to mind which are consistent with these disparate results. All three hypotheses could implicate either launching and reentry vibrations, or weightlessness, or possible other space-flight factors. The first hypothesis could implicate either vibration or weightlessness equally, the second hypothesis would lean more toward vibration effects, and the

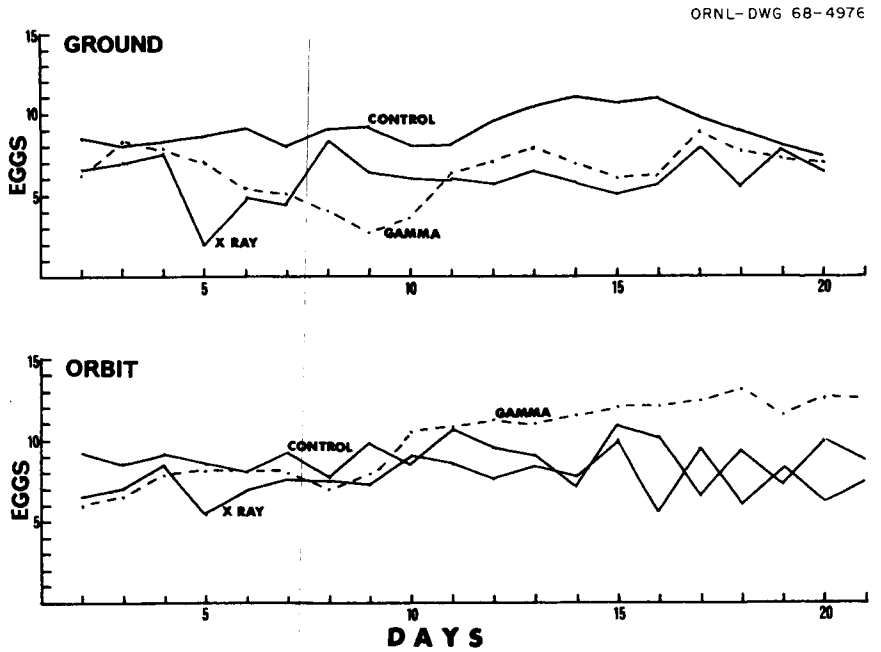

Fig. 26.56.3. Mean Daily Egg Production for Samples of Female Braconids Subsequent to Removal from the Ground Experiments Vehicle (Above) and Subsequent to Recovery from Biosatellite B (Below). The latter wasps had experienced 30 orbits, 170 miles in space. In each case curves have been drawn for unirradiated controls, for wasps receiving an acute preflight dose of 2000 r of x rays, and for wasps receiving 2667 r in a gamma-radiation exposure spread over two days. (D. S. Grosch, *Symposium on Use of Isotopes and Radiation in Entomology*, International Atomic Energy Agency, in press.)

third hypothesis would lean more toward weightlessness as the interacting agent.

The first hypothesis is that radiation-repair systems are different in meiotic and mitotic cells. In the meiotic cells the repair system is inhibited by space-flight factors; in mitotic cells the repair system is "stimulated" or unmasked to a greater extent. Perhaps in space the mitotic cells respond by being slowed in the cell cycle so that repair could be effected over a longer period of time.

The second hypothesis is that broken ends of metaphase I and prophase I meiotic chromosomes are moved further apart so that they have difficulty in rejoining, whereas broken chromosome ends in the transitional cells or in interphase oogonia are packed more closely together to be able to rejoin more readily. This hypothesis can be stated operationally as an expansion or contraction of the chromosomal rejoining sites in different cell types by space-flight factors.

The third hypothesis is that cells metabolize faster in the ground-based control setup than in the flight vehicle. A case could be made to explain the radiation synergisms and antagonisms by resorption of the eggs and their replacement by differentiating gonial cells. The sperm would of course remain equally viable under both conditions. It is difficult at this time to implicate a lower temperature in the flight vehicle as a factor, since hourly measurements of temperature in the flight vehicle and in the ground-based control setup gave almost identical readings.

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26.57 SHIFTS IN THE RECESSIVE LETHAL MUTATION SPECTRUM IN *HABROBRACON*

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Recessive lethal mutations in *Habrobracon* cause death at different stages of development. As more and more mutations have been induced by gamma radiation, x radiation, and chemical mutagens, different patterns of recessive lethal action have begun to emerge.

In general, most of the recessive lethal mutations cause death during the larval stage of development, followed in frequency by the embryonic and pupal stages (Table 26.57.1). The only exceptions are the experiments where recessive lethality is induced in sperm by brief exposures to x radiation. In these experiments the preponderance of induced recessive lethal mutations acts during the embryonic stage of development. Further, mutations induced by ethyl methane sulfonate have the highest incidence of action of all the treatments at the larval stage of development. It will be interesting to follow the pattern of action of recessive lethal mutations in additional experiments, since no obvious hypothesis accounts for the results.

26.58 THE *NEUROSPORA* EXPERIMENT (P-1037) ON BIOSATELLITE II

F. J. de Serres B. B. Webber

The initial portion of the *Neurospora* experiment in the Biosatellite II mission was successfully performed in September 1967. The spacecraft was launched on September 7 from Cape Kennedy, and 30 orbits were completed before an early calldown and recovery on September 9 over the Pacific Ocean near Hawaii. The objective of the Biosatellite II mission was to investigate the effects on living organisms of the space flight environment. Several experiments were designed to assay the genetic effects of radiation exposures from an on-board ^{85}Sr gamma radiation source during the period of weightlessness. Because of the early calldown, the radiation exposures to both the flight and simultaneous ground control vehicles were about two-thirds of those anticipated. After disassembly of the flight vehicle at Hickam Field, Honolulu, and of the ground control vehicle at Cape Kennedy, Florida, the *Neurospora* samples from both portions of the experiment were refrigerated and returned to Oak Ridge for analysis.

The genetic effects of radiation exposure during weightlessness which are being studied in *Neuro-*

Table 26.57.1. Patterns of Action of Recessive Lethal Mutations Induced in *Habrobracon* Sperm

Treatment	Exposure	Rate of Exposure	Stage of Development Where Death Occurs		
			Embryonic	Larval	Pupal
Gamma radiation	4320 r	1.11 r/min	18/78 = 0.23	20/78 = 0.26	6/78 = 0.07
	2410 r	0.62 r/min	14/177 = 0.08	23/177 = 0.13	9/177 = 0.05
	1340 r	0.34 r/min	13/235 = 0.055	15/235 = 0.064	6/235 = 0.026
	650 r	0.17 r/min	7/275 = 0.066	8/275 = 0.029	1/275 = 0.004
X radiation	2000 r	127 r/min	12/112 = 0.11	7/112 = 0.06	7/112 = 0.06
	2000 r	500 r/min	88/923 = 0.095	74/923 = 0.080	38/923 = 0.041
Ethyl methanesulfonate	0.01 *M* in sucrose solution		62/437 = 0.142	114/437 = 0.261	45/437 = 0.103
Mitomycin C	1.0 mg/ml aqueous solution		6/264 = 0.023	7/264 = 0.027	2/264 = 0.008

spora are (1) cellular inactivation and (2) the induction of recessive lethal mutations, including both those induced at two specific loci (*ad-3A* and *ad-3B*) and those induced over the entire genome. In the first phase of genetic analysis, dose-response curves for cellular inactivation and for induction of mutations in the *ad-3* region are obtained. In the second phase, genetic tests are used to detect recessive lethal mutations in the entire genome, to distinguish those recessive lethal mutations in the *ad-3* region due to point mutations from those due to chromosome deletion, and to obtain tentative identification at the molecular level of the kind of genetic alteration involved in point mutants at the *ad-3B* locus.

The data now available from this experiment indicate that there are no genetic effects of ionizing radiation under weightlessness different from those found in ground-based experiments. Included are dose-response data for (1) cellular inactivation (Fig. 26.58.1), (2) forward mutation in the *ad-3* region (Fig. 26.58.2), and (3) recessive lethal mutations in the entire genome (Table 26.58.1).

The *Neurospora* experiment on Biosatellite II is similar to that portion of the S-4 experiment on Gemini-XI involving *Neurospora* conidia on Millipore filters,[1] and present data from both of these experiments are in good agreement.

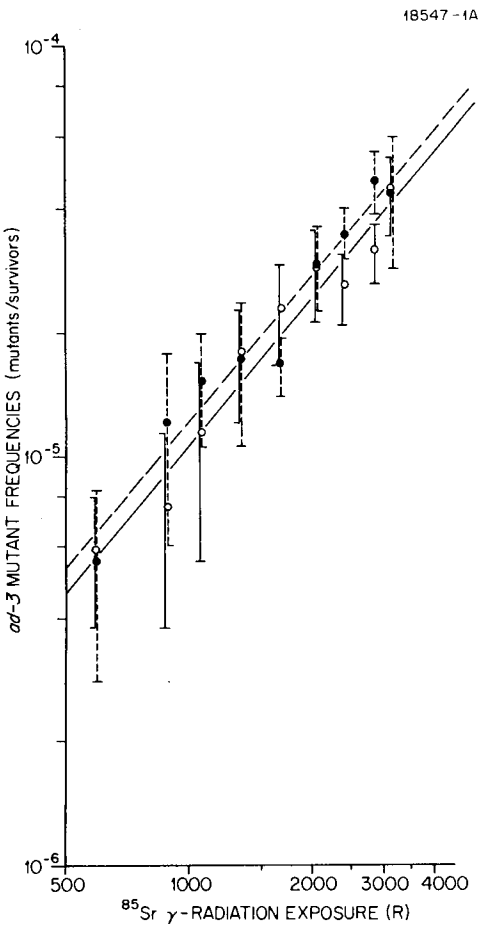

Fig. 26.58.1. Dose-Effect Curves for Survival of Heterokaryotic Conidia of a Two-Component Heterokaryon of *Neurospora crassa* Used in Experiment P-1037 on the Biosatellite II Mission. –, ● = flight samples; – –, O = ground control samples.

Reference

[1]F. J. de Serres, Ida C. Miller, Diane B. Smith, Sohei Kondo, and M. A Bender, *Biol. Div. Semi-ann. Progr. Rept. Jan. 31, 1967*, ORNL-4100, pp. 204–6.

Table 26.58.1. Results of Preliminary Tests to Detect Recessive Lethal Mutations in the Entire Genome of One Component of the *Neurospora* Heterokaryon

Spacecraft	*Neurospora* Assembly	Filter Number	Estimated Radiation Exposure (r)	Recessive Lethals Detected	Conidia Tested	Percentage of Recessive Lethals
Flight	"6000 r"	1	3116	20	198	10.1
Ground control	"6000 r"	1	3116	17	199	8.5
Flight	"6000 r"	6	2058	14	199	7.0
Ground control	"6000 r"	6	2058	13	199	6.5
Flight	"2500 r"	9	884	9	199	4.5
Ground control	"2500 r"	9	884	9	176	5.1
Flight	Aft	2	0	13	200	6.5
Ground control	Aft	2	0	8	199	4.0

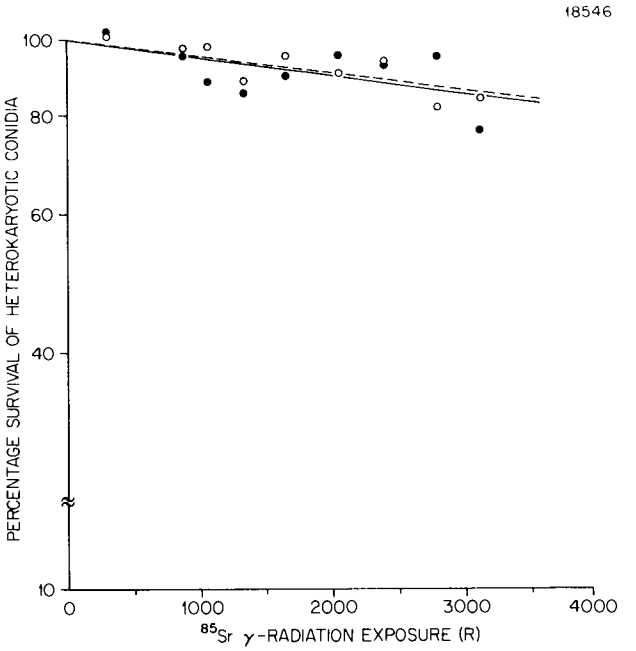

Fig. 26.58.2. Dose-Effect Curves for Induction of Recessive Lethal Mutations at Specific Loci (*ad3A* and *ad3B*) in Heterokaryotic Conidia of a Two-Component Heterokaryon of *Neurospora crassa* Used in Experiment P-1037 on the Biosatellite II Mission. —, ● = flight samples; – –, O = ground control samples.

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