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SEMIANNUAL PROGRESS REPORT

FOR PERIOD ENDING JANUARY 31, 1967

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BIOLOGY DIVISION
SEMIANNUAL PROGRESS REPORT
For Period Ending January 31, 1967

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

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JUNE 1967

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ORNL-1393	Period Ending August 10, 1952
ORNL-1456	Period Ending November 10, 1952
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ORNL-2997	Period Ending August 15, 1960
ORNL-3095	Period Ending February 15, 1961
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ORNL-3352	Period Ending August 15, 1962
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
ORNL-3768	Period Ending February 15, 1965
ORNL-3853	Period Ending July 31, 1965
ORNL-3922	Period Ending January 31, 1966
ORNL-3999	Period Ending July 31, 1966

Contents

PUBLICATIONS AND LECTURES 1

Publications 1
Lectures 11
Visiting Lecturers 25
Scheduled Speakers for Professional Meetings, Spring–Summer 1967 29

FOREIGN TRAVEL 30

MEETINGS AND CONFERENCES 32

1966 – Meeting on the Use of DNA or RNA to Supplement the Host Genome, Perhaps Using Viruses as Vectors 32
Fall Meeting of Biomedical Program Directors 32
Symposium on Enzymatic Aspects of Metabolic Regulation 32
Bone Marrow Conference 33
Annual Information Meeting 33

1967 – Twentieth Annual Biology Research Conference 33
Biomedical Program Directors Meetings 35
Bone Marrow Conferences 35
2nd International Conference on Cell Synchrony 36
Third Conference on Blood Platelets 36
Eighth Annual Symposium of the International Society for Cell Biology 36
International Symposium on Basic Mechanisms in Photobiology 36

EDUCATIONAL ACTIVITIES 37

Graduate Courses Taught 37
Lectures Presented in Courses 37
Courses Taken 38

DIVISION MEMBERS WORKING IN OTHER LABORATORIES 38

Leave of Absence 38
Collaborative Research 39

CYTOLOGY AND GENETICS 40

1.1 Photoreactivation of Pyrimidine Dimers in *Paramecium aurelia* DNA – **Betsy M. Sutherland, R. B. Setlow** 40

1.2 The Repair of UV Damage in *Tetrahymena pyriformis* GL-C – **G. L. Whitson, A. A. Francis, W. L. Carrier** 41

1.3 Cyclic Changes of the Alcohol-Soluble Carbohydrates in Synchronized *Tetrahymena* – **G. L. Whitson, A. A. Francis, J. G. Green, D. D. Willis** 42

1.4 The Minimum Size of the Substrate for the Photoreactivating Enzyme – **Jane K. Setlow, F. J. Bollum** 42

1.5 The Relationship Between Cytochrome b_2 and Photoreactivating Enzyme – **Jane K. Setlow, Dianne C. Brown** 42

1.6 Alcohol Dehydrogenase and Invertase in Stationary and Logarithmically Growing Yeast – **M. E. Boling, Jane K. Setlow** 43

1.7 Localization of Photoreactivating Enzyme in a Bacterial Cell – **Amikam Cohen, Jane K. Setlow, H. I. Adler** 43

1.8 Transplantation Antigens of Mouse Spleen Cells – **R. A. Popp, N. G. Anderson, Diana M. Popp, W. S. McEwen** 43

1.9 Possible Identity of Rho and "R" Antigens in Mice – **Diana M. Popp, G. D. Snell** 44

1.10 Patterns of Puff Development in Rhynchosciara – **M. Ellen Mattingly, C. L. Parker** 45

1.11 Heterozygosity in Puffs and Chromosomes of *Sciara ocellaris* – **A. L. P. Perondini** 46

1.12 Nucleic Acid Synthesis in Polytene Chromosomes of Rhynchosciara – **L. C. G. Simoes** 46

FUNGAL GENETICS 48

2.1 Identification of the Genetic Alterations in X-Ray-Induced *ad-3B* Mutants of *Neurospora crassa* at the Molecular Level – **H. V. Malling, F. J. de Serres** 48

2.2 Enzyme Assays for the *ad-3* Mutants of Neurospora – **C. R. Fisher** 50

2.3 The Effect of Photoreactivation on UV-Induced Inactivation of Heterokaryotic Conidia and Forward Mutation in the *ad-3* Region of *Neurospora crassa* – **B. J. Kilbey, F. J. de Serres** 50

2.4 Genetic Modification of Crossover Frequency and the Formation of Aberrant Marker Ratios in the Asci of *Neurospora* – **J. R. Baylis, Jr., F. J. de Serres** 52

2.5 Genetic Analyses of Revertants of *ad-3B* Mutants in *Neurospora crassa* – **H. V. Malling** 52

DROSOPHILA CYTOLOGY AND GENETICS 54

3.1 Studies of Temperature-Induced Recombination in the Oocyte of *Drosophila melanogaster* – **Rhoda F. Grell, J. W. Day** 54

3.2 Mutants Affecting Meiosis in Drosophila – **D. L. Lindsley, L. Sandler, B. Nicoletti, G. Trippa** 55

3.3 Meiotic Behavior of Tandem Acrocentric Compound *X* Chromosomes in Female *Drosophila melanogaster* – **J. R. Merriam** 58

3.4 Selection of Gene Mutations Causing the Absence of Alcohol Dehydrogenase in *Drosophila melanogaster* – **E. H. Grell** 59

3.5 Analysis of a Small Duplication – **D. R. Parker** 60

3.6 Translocations and Recombination – **J. H. Williamson** 61

YEAST, HYMENOPTERA, AND PHAGE GENETICS 62

4.1 Reversion Rate Studies of the tr_{1-1} Mutant – **R. C. von Borstel, C. M. Steinberg** 62

4.2 Electrophoretic Patterns of Xanthine Dehydrogenase in Two Species of Habrobracon – **R. H. Smith, L. R. Valcovic** 64

4.3 The Intersex Locus in Habrobracon – **M. B. Baird, R. C. von Borstel** 65

4.4 Native DNA with Priming Activity in Amphibian Chromosomes – **R. C. von Borstel, O. L. Miller, Jr., F. J. Bollum** 66

4.5 Growth Inhibition Without Inhibition of Differentiation by Ribonuclease in Chick Embryos – **S. K. Brahma, R. C. von Borstel** 66

HUMAN CYTOGENETICS 70

5.1 Chromosome Aberration Yield as a Function of Time of Fixation of Human Leukocyte Cultures – **M. A Bender** 70

5.2 High Chromosome Breakage Rates in Leukocytes of a Normal Female – **P. Carolyn Gooch, C. L. Fischer** 72

5.3 The Fate of Ultraviolet-Light-Induced Thymine-Containing Dimers in Human Cells Grown in Vitro – **J. D. Regan, J. E. Trosko** 73

CELL GROWTH AND DIFFERENTIATION 75

6.1 Cell Generation Time in Various Stages of Lens Regeneration – **Sara Eisenberg, Tuneo Yamada** 75

6.2 Autoradiographic Studies of DNA Synthesis During the Development and Differentiation of Chick Lens – **S. P. Modak, Georgia Morris, Marion E. Roesel** 76

6.3 Immunoelectrophoretic Analyses of the Purity of *Rana pipiens* γ-Crystallin Samples – **D. S. McDevitt, Isaura Meza** 78

6.4 RNA Synthesis During Lens Regeneration – **Egidio Puccia, D. H. Reese, Tuneo Yamada** 79

6.5 The Membranous Component of *Triturus* Oocyte Nucleoli – **O. L. Miller, Jr.** 80

6.6 Bulk Isolation of Amphibian Oocyte Nuclei – **O. L. Miller, Jr., George Gassner III** 81

6.7 Ultrastructural Studies on the Centriole Adjunct in Developing Sperm of the Wasp *Mormoniella vitripennis* (Walker) – **George Gassner III** 81

6.8 Crustacean Lipovitellin: Isolation and Characterization of the Major High-Density Lipoprotein from the Eggs of Decapods – **R. A. Wallace, Susan L. Walker** 82

6.9 Vitellogenesis in the Horseshoe Crab, *Limulus polyphemus* – **J. N. Dumont** 84

6.10 Comparative Chromatography of the Yolk Proteins of Teleosts – **D. W. Jared, R. A. Wallace** 85

6.11 A Lipoprotein from Hemolymph and Oocytes of the Blue Crab – **Marilyn S. Kerr** 88

6.12 Studies on Ribosomal Degradation in Lens Cells – **W. F. Zapisek, John Papaconstantinou** 89

6.13 The Stabilization of mRNA in Lens Epithelial Cells – **J. A. Stewart, John Papaconstantinou** 90

6.14 Immunofluorescent Localization of α-, β-, and γ-Crystallins in Bovine Lens Cells During Growth and Differentiation – **R. E. Shore, John Papaconstantinou** 91

6.15 An Analysis of the Pulse-Labeled RNA in Bovine Lens Epithelial Cells and Fiber Cells – **John Papaconstantinou, Emilia M. Julku** 93

6.16 The Synthesis of Lens Proteins by Epithelial Cells in Tissue Culture – **John Papaconstantinou, J. W. Carr** 94

6.17 Studies on the Subunit Composition of Bovine Lens α-Crystallins – **Winnie G. Palmer, John Papaconstantinou** 95

RADIATION MICROBIOLOGY AND MICROBIAL GENETICS 97

7.1 Acridine Effects in Irradiated *Neurospora crassa* – **C. E. Terry** 97

7.2 Correlation Between Increased Radiation Resistance and Increased Cell Size of the Bacterium *Escherichia coli* – **D. E. Axelrod, H. I. Adler** 98

7.3 A Mutation Affecting the Size, Shape, and Radiation Resistance of *Escherichia coli* K-12 – **H. I. Adler, Alice A. Hardigree** 98

7.4 A New Cell Division Mutant of *Escherichia coli* – **H. I. Adler, Amikam Cohen, Alice A. Hardigree, D. P. Allison** 99

7.5 Genetic Transfer to Minicells of *Escherichia coli* K-12 – **Amikam Cohen, D. P. Allison, Roy Curtiss III** 99

7.6 Energy Requirements for Specific Pair Formation During Conjugation in *Escherichia coli* K-12 – **Roy Curtiss III, D. R. Stallions** 101

7.7 Macromolecular Syntheses and Energy Metabolism During Conjugation in Hfr $\times$ F^- Matings of *Escherichia coli* K-12 – **Roy Curtiss III, Jane A. Mays, D. R. Stallions** 101

7.8 Mechanism of Chromosome Mobilization and Transfer by F^+ Donors of *Escherichia coli* K-12 – **Roy Curtiss III, D. R. Stallions, Jane A. Mays, Janet Renshaw** 102

7.9 Localization of Genes Specifying tRNA Species in *Escherichia coli* K-12 – **G. J. Stine, Annie S. Angel, Roy Curtiss III** 103

RADIATION IMMUNOLOGY 104

8.1 Variation in Blast Cell Cycle During Log-Stationary Phase of Secondary Antibody Response Initiated in in Vivo Diffusion Chamber Cultures – **Toshihiko Sado** 104

8.2 Characterization of Antibody-Synthesizing Cultures – **D. C. Vann** 105

8.3 Synthesis of Antibody by Spleen Cells After Exposure to Kiloroentgen Doses of Ionizing Radiation – **Takashi Makinodan, Paul Nettesheim, Toshiteru Morita** 106

8.4 The Involvement of the Spleen in Senescence and Death in an Inbred Strain of Mice – **J. F. Albright, Takashi Makinodan** 107

8.5 Primary and Secondary Antibody Response by Mice and Their Spleen Cells to Human Gamma Globulin – **A. E. Sassen, E. H. Perkins** 108

8.6 Erythropoietic Potentialities of Cells of the Embryonic Yolk Sac – **J. F. Albright, Julia W. Deitchman** 108

8.7 Sheep Stroma as an Immunoabsorbent – **R. A. Brown, W. R. Somers, Takashi Makinodan** 109

8.8 Effect of X Irradiation on RNA Metabolism of PHA-Transformed Lymphocytes in Vitro – **Hiroo Yamamoto** 109

MAMMALIAN GENETICS 110

9.1 Effect of the Interval Between X Irradiation and Conception on Mutation Frequency in Female Mice – **W. L. Russell, Elizabeth M. Kelly** 110

9.2 Chemically Induced Dominant Lethals in Male Mice – **U. H. Ehling, R. B. Cumming, H. V. Malling, Sandra W. Huff** 111

9.3 Analysis of Genetic Changes in a Short Region of Linkage Group II of the Mouse – **Liane B. Russell, Martha M. Larsen, Kathren F. Stelzner, Mary H. Steele, H. M. Thompson, Jr.** 113

9.4 ^{3}H-Uridine Labeling of Mouse Oocytes – **E. F. Oakberg, Evelyn C. Lorenz, Roselynn A. Dynesius, Diane W. Slover** 115

GENERAL PHYSIOLOGY 117

10.1 The Control of Cell Division in *Escherichia coli* by Substances in Cell Extracts – **W. D. Fisher, H. I. Adler, F. W. Shull, Jr.** 117

MAMMALIAN RECOVERY 118

11.1 The Effect of Hypoxia on Mortality from Secondary Disease – **C. C Congdon, M. L. Simmons, R. E. Toya, Sr.** 118

11.2 Serine-3-^{14}C Metabolism in Irradiation Chimeras: Respiration Pattern Analysis – **A. L. Kretchmar, C. E. Phipps** 119

11.3 Parasites in the Alimentary Tract of Radiation Chimeras – **W. H. McArthur** 121

11.4 Erythropoiesis in the Mouse – **L. H. Smith, T. W. McKinley, Jr.** 121

11.5 Further Studies on Modification of Poor Growth of Parental Marrow Cells in F_1 Hybrid Mice – **Joan Wright Goodman, H. Brownell Wheeler** 122

11.6 A Facility Designed for Pathogen-Free Inbred Mouse Production – **M. L. Simmons, L. P. Wynns, E. E. Choat** 123

11.7 Distribution of Antibody to Viruses in Experimental Mice – **R. W. Tennant, Roberta K. Reynolds, K. R. Layman** 125

11.8 The Isolation of *Salmonella poona* and a Nonmotile Variant from Laboratory Mice – **J. A. Franklin, C. B. Richter** 126

PATHOLOGY AND PHYSIOLOGY 127

12.1 Preliminary Data on the Longevity and Leukemia Incidence in Mice of the Barrier-Sustained RFM/UnF Subline to Be Used in the Low(Medium) Level Experiment – **All Members of Pathology-Physiology Section** 128

12.2 Polyploidy and Maturation of Megakaryocytes – **T. T. Odell, Jr., C. W. Jackson, Rebecca S. Reiter** 128

12.3 Estimation of the Frequency Distribution of Megakaryocytes in Ploidy Classes – **D. G. Gosslee, T. T. Odell, Jr., C. W. Jackson** 129

12.4 Influence of the Microbial Environment on the Development of Myeloid Leukemia in X-Irradiated RFM Mice – **H. E. Walburg, Jr., G. E. Cosgrove** 130

12.5 Methylcholanthrene-Induced Myeloid Leukemia in Germfree RFM Mice – **H. E. Walburg, Jr., G. E. Cosgrove** 131

12.6 Some Characteristics of Transplantable Myxofibrosarcomas – **R. L. Tyndall, R. C. Brown, Ernestine Teeter, J. A. Otten** 132

12.7 Altered Esterase Zymograms Associated with Leukemia Virus Infection – **R. L. Tyndall, R. C. Allen, Dorothy J. Moore** 132

12.8 The Use of Germfree Mice to Monitor the Microbiological Environment – **R. C. Allen, R. T. Jones, L. J. Serrano, J. H. Chandler, Jr., G. W. Fortner** 133

PLANT PHYSIOLOGY AND PHOTOSYNTHESIS 135

13.1 Growth Promotion and Inhibition Resulting from Reirradiation of Gamma-Plantlets – **A. H. Haber, Pat C. Kerr, D. E. Foard** 135

13.2 Actions of Gamma Radiation on Senescence and Death in Excised Wheat Leaf Tissue After Normal Cessation of Cell Growth and Division – **A. H. Haber, W. D. Willis** 136

13.3 Effects of Acute Gamma Irradiation on Subsequent Changes in Chloroplast Ultrastructure in Excised, Nonilluminated Wheat Leaf Tissue – **Patricia L. Walne, Sarah A. Angel, A. H. Haber** 137

13.4 Evidence for Histologic Programming of Senescence Separate from Cell Growth and Division – **D. E. Foard, Sarah A. Angel, Carolyn P. Richardson** 138

13.5 Photosynthesis – **W. A. Arnold, W. F. Bertsch, J. R. Azzi, L. W. Jones, R. M. Pearlstein, J. B. Davidson** 138

CELL PHYSIOLOGY 141

14.1 Freezing of *Escherichia coli* – **Peter Mazur, Janice J. Schmidt** 141

14.2 Freezing of Bacteriophage T4B – **S. P. Leibo, Peter Mazur** 142

14.3 Correlation Between Water Activity and Survival of Bacteriophage T4B – **S. P. Leibo, Peter Mazur** 143

14.4 Survival of Supercooled Yeast – **Peter Mazur, Janice J. Schmidt, Margaret R. Kasschau** 144

NUCLEIC ACID CHEMISTRY 146

15.1 Nucleoside Sequences in Transfer RNA – **All Members of Nucleic Acid Chemistry Section** 146

15.2 Oligonucleotide Interactions – **S. K. Niyogi** 146

NUCLEIC ACID ENZYMOLOGY 148

16.1 Purification of T2 Messenger RNA – **Elliot Volkin, W. S. Riggsby** 148

16.2 Repair of Parental T5 Bacteriophage DNA After Infection of *Escherichia coli* – **R. K. Fujimura, Elliot Volkin** 149

16.3 An Endonuclease from T2-Infected *Escherichia coli* – **C. G. Mead** 149

16.4 Mitochondrial Specific Transfer RNA's and Aminoacyl-RNA Synthetases – **W. E. Barnett, J. L. Epler, D. H. Brown, Diane J. Goins** 150

16.5 The Use of the Cetyltrimethylammonium Cation in Nucleotide Sequence Analyses – **J. X. Khym, Mayo Uziel** 150

16.6 Biosynthesis of Adenine Derivatives by Nauplii of *Artemia salina* – **F. J. Finamore, Rose P. Feldman** 151

CHEMICAL PROTECTION AND ENZYME CATALYSIS 152

17.1 Chemical Protection – **D. G. Doherty, Margaret A. Turner** 152

17.2 Glyoxalase I – **D. G. Doherty, Margaret A. Turner** 152

ENZYMOLOGY 153

18.1 Comparison of Mitochondrial and Cytoplasmic tRNA from *Neurospora crassa* – **D. H. Brown, G. David Novelli, W. E. Barnett** 153

18.2 Biochemical Processing. Large-Scale Fermentation – **E. F. Phares, Mary V. Long, G. David Novelli** 154

18.3 Studies on Antibody Biosynthesis – **C. J. Wust** 154

18.4 Studies on RNA During the Immune Response – **C. J. Wust, D. G. Ridner** 155

18.5 Activity Studies on *E. coli* Polysomes – **Peter Pfuderer, Donna G. Shaffer** 156

18.6 Studies of the Binding of RNA Polymerase to Polynucleotides – **Audrey L. Stevens** 156

18.7 The Binding of $tRNA^{Phe}$ to Phenylalanyl-RNA Synthetase – **M. P. Stulberg, K. R. Isham** 157

18.8 Effect of Methylation on Phenylalanine Transfer RNA from *Escherichia coli* – **L. R. Shugart, M. P. Stulberg** 157

18.9 Large-Scale Isolation of Phenylalanyl-RNA Synthetase from *E. coli* B – **R. L. Pearson, M. P. Stulberg** 158

18.10 Optical Studies of the Structure and Function of tRNA – **M. P. Stulberg, J. W. Longworth** 158

18.11 Isoenzymes: Their Regulation and Genetic Control – **K. B. Jacobson, J. B. Murphy** 158

18.12 Further Studies on the Aminoacylation of *E. coli* tRNA Fractions by *Neurospora crassa* Phenylalanine tRNA Synthetases – **Virginia L. Holten, K. B. Jacobson** 159

BIOCHEMICAL REGULATION 160

19.1 Enzyme Turnover in Vivo: Effects of Inhibitors of RNA and Protein Synthesis – **F. T. Kenney, Joseph Kendrick, L. E. Roberson** 160

19.2 Hormonal Repression of Enzyme Synthesis in "Pathogen-Free" Rats – **F. T. Kenney, Joseph Kendrick, L. E. Roberson** 161

19.3 Preparation and Properties of Fetal Rat Liver Polyribosomes – **W. D. Wicks** 161

19.4 Hormonal Control of Enzyme Synthesis in the Isolated, Perfused Rat Liver – **C. B. Hager, F. T. Kenney, G. R. Holloway** 162

19.5 RNA Synthesis in Rat Liver Under the Action of Insulin – **Claudio Pellegrino, F. T. Kenney** 163

MICROBIOLOGY 165

20.1 Kinetics of Malic-Lactic Transhydrogenase – **M. I. Dolin** 165

BIOPHYSICS 166

21.1 Role of the λ Repressor – **R. A. Weisberg** 166

21.2 Studies of Plastid Ribosomes – **Ann B. Jacobson, Peggy P. Hester** 167

21.3 Satellite DNA's of Crustacea – **Dorothy M. Skinner, L. L. Triplett** 167

21.4 UV-Irradiation Studies of Naturally Occurring "Poly d(A-T)" – **Dorothy M. Skinner, R. B. Setlow** 169

21.5 Interruptions in the Chromosomal DNA of a Slime Mold – **R. A. McGrath, R. W. Williams** 169

21.6 Repair of Damaged DNA in Several Strains of X-Irradiated *Escherichia coli* – **Sandra J. Roland, R. A. McGrath** 170

21.7 Enzymes Involved in Dimer Excision in Vitro – **W. L. Carrier, Janet R. McGrath, R. B. Setlow** 170

21.8 Chain Breaks and Cross Links in DNA Irradiated in Vivo with Ultraviolet Light – **R. B. Setlow, R. W. Williams** 171

21.9 Excision of Pyrimidine Dimers in *Escherichia coli* – **R. B. Setlow, W. L. Carrier** 171

21.10 Direct Demonstration that Yeast Photoreactivating Enzyme and Light Monomerize Thymine-Containing Dimers in UV-Irradiated DNA – **J. S. Cook** 172

21.11 Distribution of Photoreactivating Enzymes – **J. S. Cook, Janet R. McGrath** 172

21.12 Delayed Response in the Photoreactivation of Tryptophanase Inducibility in *Escherichia coli* B – **P. A. Swenson** 173

21.13 Effects of Ultraviolet Irradiation on Respiration of Radiation-Resistant and Sensitive Strains of *Escherichia coli* – **Barbara A. Hamkalo, P. A. Swenson** 173

21.14 Action Spectra for Survival and Photoproduct Production in *B. megaterium* Spores – **J. E. Donnellan, Jr., J. L. Otis, R. S. Stafford** 174

21.15 The Ultraviolet Photochemistry of Germinating Spores – **R. S. Stafford, J. E. Donnellan, Jr.** 174

21.16 The Biological Significance of a New Photoproduct Formed in DNA at 77°K – **R. O. Rahn, Jane K. Setlow** 175

21.17 Nature of Photoproduct Formed in DNA at 77°K – **R. O. Rahn, J. L. Hosszu** 175

21.18 Pyrimidine Dimer Formation in Poly T at 77°K – **R. O. Rahn, J. L. Hosszu** 176

21.19 Elemental Composition of Tissues as a Limiting Factor in the Specification of Dose of Ionizing Radiation – **M. L. Randolph** 176

COOPERATIVE PROGRAMS 178

AEC-NIH Chemical Carcinogenesis 178

22.1 Studies with Certain Oncogenic and Plant Viruses – **Stanfield Rogers** 179

22.2 Ethionine Carcinogenesis – **Beryl Ortwerth, G. David Novelli** 180

22.3 Studies on Amino Acid Activation in Mouse Plasma Cell Tumors – **Wen-Kuang Yang, G. David Novelli, C. J. Wust** 180

22.4 Physiological Parameters of L-M Cells Propagated in Vivo and in Vitro – **Kiki B. Hellman, Alfred Hellman, R. B. Cumming, G. E. Cosgrove, G. David Novelli** 181

22.5 Leucyl-tRNA from T2-Infected *E. coli* B – **L. C. Waters, Elliot Volkin, G. David Novelli** 181

22.6 Studies on the Purification and Characteristics of tRNA Pyrophosphorylase – **Audrey N. Best, G. David Novelli** 182

22.7 Isolation of the Product Formed by the Soluble Amino Acid Incorporating System – **Lawrence Rosen, Shigemi Iiyama, G. David Novelli** 182

22.8 Preliminary Observations on the Combined Late Somatic Effects of X Rays and Triethylene Melamine in Mice – **J. W. Conklin, W. D. Gude** 183

22.9 Leukemogenic Effects of Methylcholanthrene on the Mouse Thymus – **Lou C. Satterfield, Mildred G. Hayes, A. W. Craig** 184

22.10 Selection for Genetic Variants in Mammalian Somatic Cells in Vitro – **E. H. Y. Chu, E. Virginia Merriam, Patricia A. Brimer** 185

22.11 Comparison of Mutation Induction by X Rays, Ultraviolet Radiation, Triethylene Melamine, and Nitrosoguanidine – **R. F. Kimball, Barbara R. Bussell, E. G. Bailiff, Stella W. Perdue** 186

22.12 Tales of Tyrosine in Polymers – **J. W. Longworth** 187

22.13 X-Ray Crystallography – **J. R. Einstein, C. H. Wei** 187

22.14 Incorporation of Heavy Atoms into Proteins – **F. C. Hartman** 188

22.15 The Relationship Between Carcinogenicity and Mutagenicity of Ethylating Compounds – **H. V. Malling, F. J. de Serres** 188

22.16 Inhalation Carcinogenesis – **D. G. Doherty, J. O. Brick, M. G. Hanna, Jr., Alfred Hellman, Paul Nettesheim, R. F. Newell** 190

22.17 Reduced Immune Potential of Aged Mice: Significance of Morphologic Changes in Lymphatic Tissue – **M. G. Hanna, Jr., Paul Nettesheim, Takashi Makinodan** 192

22.18 Subcellular Fractionation Studies of ^{125}I-Soluble Antigen Retention in Spleen Lymphatic Tissue and Liver – **Mary W. Francis, M. G. Hanna, Jr., W. D. Fisher** 194

22.19 Ultrastructural Studies of Antigen Localization in Spleen Lymphatic Tissue – **A. K. Szakal, M. G. Hanna, Jr.** 195

22.20 Factors Influencing the X-Ray Recovery of Secondary Antibody-Forming Potential – **Paul Nettesheim, Mary Lou Williams** 196

22.21 Aminoacyl sRNA Levels in Viral and Nonviral Infected Mammalian Cells – **Alfred Hellman, L. J. Wopschall, D. H. Martin** 198

22.22 In Vivo Protein Damage to *Escherichia coli* by Benzo(a)pyrene with 355 mμ Illumination – **A. P. Harrison, Jr., Vivian E. Raabe** 198

22.23 Hormonal Regulation of Enzyme Synthesis in Suspension Cultures of the Reuber Hepatoma – **J. R. Reel, F. T. Kenney, J. D. Regan** 199

22.24 Tyrosine Transaminase in Regenerating Liver – **Claudio Pellegrino, F. T. Kenney** 200

22.25 Regulation of Enzyme Synthesis by Hydrocortisone in Organ Cultures of Fetal Rat Liver – **W. D. Wicks, J. D. Regan** 200

AEC-NASA Space Biology 202

22.26 The *Neurospora* Experiment on Biosatellite A – **F. J. de Serres, B. B. Webber** 202

22.27 The Flight of Biosatellite A – **R. C. von Borstel, D. H. Grosch, Joan W. Reel, R. H. Smith, L. R. Valcovic, Martha S. Jones, Anna R. Whiting, M. B. Baird, Margaret L. Haye, R. L. Amy, Katherine T. Cain** 203

22.28 Habrobracon Survival and Dominant Lethality in the Ground-Based Control for the 301 Walk-Thru Test of the Biosatellite Experiment – **R. H. Smith, Katherine T. Cain, Anna R. Whiting, Joan W. Reel, R. C. von Borstel, Martha S. Jones, L. R. Valcovic, Margaret L. Haye, M. B. Baird** 203

22.29 The Gemini XI S-4 Experiment. II. The Neurospora Experiment – **F. J. de Serres, Ida C. Miller, Diane B. Smith, Sohei Kondo, M. A Bender** 204

ORGANIZATIONAL CHANGES

Alexander Hollaender retired as Division Director December 31, 1966. Dr. Hollaender is remaining with the Division as Senior Research Adviser.

J. L. Liverman, formerly an Associate Director of the Biology Division, was appointed an Assistant Director of the Oak Ridge National Laboratory. This appointment became effective January 1, 1967. Reporting to Dr. Liverman are the Biology and Health Physics Divisions and the Civil Defense Research Project.

R. F. Kimball was appointed Director of the Biology Division, effective January 1, 1967. Prior to this appointment Dr. Kimball was head of the Division's Cytology and Genetics section.

S. F. Carson, formerly an Associate Director, was appointed Deputy Director of the Biology Division, also effective January 1, 1967.

Publications and Lectures

PUBLICATIONS

During this report period (August 1, 1966–January 31, 1967), 373 papers, abstracts, and book reviews by members of the Division have been published or are in press. Listed below are the 120 published papers and reports, and books edited, by Division members for the current period. There were 90 published manuscripts for the corresponding period in 1965–1966, and 88 for the period February 1–July 31, 1966. The 28 published abstracts, 6 published book reviews, and 219 items in press are not listed.

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Allen, R. C., and G. R. Jamieson	An automated microdensitometer for the quantitation of pherograms and zymograms in acrylamide gels	*Anal. Biochem.* **16**, 450–56 (1966)
Allen, R. C., and Dorothy J. Moore	A vertical flat-bed discontinuous electrophoresis system in polyacrylamide gel	*Anal. Biochem.* **16**, 457–65 (1966)
Anderson, N. G. (editor)	*The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (526 pp.)	*Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Bethesda, Maryland, 1966

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Anderson, N. G.	Introduction	Pp. 1–5 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
	An introduction to particle separations in zonal centrifuges	*Ibid.*, pp. 9–39
	Molecular anatomy: Next major science programme?	*Science J.* **3**, 35–41 (1967)
	Zonal centrifuges and other separation systems	*Science* **154**, 103–12 (1966)
Anderson, N. G., H. P. Barringer, J. W. Amburgey, Jr., G. B. Cline, C. E. Nunley, and A. S. Berman	Continuous-flow centrifugation combined with isopycnic banding: Rotors B-VIII and B-IX	Pp. 199–216 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Anderson, N. G., H. P. Barringer, E. F. Babelay, C. E. Nunley, M. J. Bartkus, W. D. Fisher, and C. T. Rankin, Jr.	The design and operation of the B-IV zonal centrifuge system	*Ibid.*, pp. 137–64
Anderson, N. G., H. P. Barringer, Nakwon Cho, C. E. Nunley, E. F. Babelay, R. E. Canning, and C. T. Rankin, Jr.	The development of low-speed "A" series zonal rotors	*Ibid.*, pp. 113–36
Anderson, N. G., G. B. Cline, R. M. Jamison, and W. W. Harris	Semiannual Contract Progress Report to the National Institute of Allergy and Infectious Diseases	ORNL-3942
Anderson, N. G., J. G. Green, and Peter Mazur	Centrifugal freezing. I. A system for rapid freezing of aqueous cell suspensions	Pp. 415–30 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Anderson, N. G., W. W. Harris, A. A. Barber, C. T. Rankin, Jr., and E. L. Candler	Separation of subcellular components and viruses by combined rate- and isopycnic-zonal centrifugation	*Ibid.*, pp. 253–83

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Arnold, W. A.	Light reaction in green plant photosynthesis: A method of study	*Science* **154**, 1046–49 (1966)
Azzi, J. R.	The delayed light and fluorescence emission spectra for green, blue-green, red algae, and nonphotosynthetic mutants	ORNL-TM-1534
Barber, A. A., T. W. Bartlett, and B. H. Levedahl	Isolation of paramylon from *Euglena gracilis* var *bacillarus* SML-1 by combined rate- and isopycnic-zonal centrifugation	Pp. 303–16 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Barber, A. A., W. W. Harris, and N. G. Anderson	Isolation of native glycogen by combined rate-zonal and isopycnic centrifugation	*Ibid.*, pp. 285–302
Barber, A. A., C. T. Rankin, Jr., and N. G. Anderson	Lipid peroxidation in rat tissue particulates separated by zonal centrifugation	*Ibid.*, pp. 333–44
Barber, E. J.	Calculation of density and viscosity of sucrose solutions as a function of concentration and temperature	*Ibid.*, pp. 219–39
Barber, Mary Lee S., and R. E. Canning	Extraction of contractile proteins from myofibrils prepared by rate-zonal centrifugation	*Ibid.*, pp. 345–59
Barringer, H. P.	The design of zonal centrifuges	*Ibid.*, pp. 77–111
Barringer, H. P., N. G. Anderson, and C. E. Nunley	Design of the B-V continuous-flow centrifuge system	*Ibid.*, pp. 191–98
Barringer, H. P., N. G. Anderson, C. E. Nunley, K. T. Ziehlke, and W. S. Dritt	Zonal rotors with removable seals: Rotors B-X and B-XI	*Ibid.*, pp. 165–74
Bender, M. A	Synergistic Effect of Zero-G and Radiation on White Blood Cells: An Experiment for the Gemini III Manned Space Flight, Annual Report for Period Ending June 30, 1965	ORNL-TM-1550
Bender, M. A, and P. Carolyn Gooch	Somatic chromosome aberrations induced by human whole-body irradiation: The "Recuplex" criticality accident	*Radiation Res.* **29**, 568–82 (1966)
Bender, M. A, P. Carolyn Gooch, and Sohei Kondo	Experiment S-4, zero *g* and radiation on blood during Gemini III	Pp. 217–36 in *Manned Space Flight Experiments Symposium: Gemini Missions III and IV, Held in Washington, D. C., Oct. 18–19, 1965*, National Aeronautics and Space Administration, 1965

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Berman, A. S.	Theory of centrifugation: Miscellaneous studies	Pp. 41–76 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Bishop, Barbara S.	Digital computation of sedimentation coefficients in zonal centrifuges	*Ibid.*, pp. 175–88
Boling, M. E., and Jane K. Setlow	The resistance of *Micrococcus radiodurans* to ultraviolet radiation. III. A repair mechanism	*Biochim. Biophys. Acta* **123**, 26–33 (1966)
Brahma, S. K.	An autoradiographic study of RNA synthesis during primary embryonic induction	*J. Embryol. Exptl. Morphol.* **16**, 203–10 (1966)
Brewen, J. G., and Gregorio Olivieri	The kinetics of chromatid aberrations induced in Chinese hamster cells by tritium-labeled thymidine	*Radiation Res.* **28**, 779–92 (1966)
Browne, L. E., J. L. Noey, Pat C. Kerr, and A. H. Haber	Air-turbulence and temperature gradients reduced in plant growth chambers by small-hole diffuser walls	*Bioscience* **17**, 37 (1967)
Cameron, I. L., G. B. Cline, G. M. Padilla, O. L. Miller, Jr., and P. A. Van Dreal	Polysomes from *Tetrahymena* following pyrimidine deprivation and replacement	Pp. 361–71 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Cameron, I. L., and W. D. Fisher	Separation of acid-soluble nucleotides from yeast with the nucleotide analyzer	*Ibid.*, pp. 441–46
Caro, L. G., and Maria Schnös	The attachment of the male-specific bateriophage fl to sensitive strains of *Escherichia coli*	*Proc. Natl. Acad. Sci. U.S.* **56**, 126–32 (1966)
	The bacteriophage P1kc and its DNA	*Microbial Genet. Bull.* **24**, 6 (1966)
Carrier, W. L., and R. B. Setlow	Excision of pyrimidine dimers from irradiated DNA in vitro	*Biochim. Biophys. Acta* **129**, 318–25 (1966)
Cattanach, B. M.	Chemically induced mutations in mice	*Mutation Res.* **3**, 346–53 (1966)
	Induction of paternal sex-chromosome losses and deletions and of autosomal gene mutations by the treatment of mouse post-meiotic germ cells with triethylenemelamine	*Mutation Res.* **4**, 73–82 (1967)
Chandley, Ann C.	Studies on oogenesis in *Drosophila melanogaster* with ^{3}H-thymidine label	*Exptl. Cell Res.* **44**, 201–15 (1966)
Childs, H. E., Jr., and G. E. Cosgrove	A study of pathological conditions in wild rodents in radioactive areas	*Am. Midland Naturalist* **76**, 309–24 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Cho, Nakwon, H. P. Barringer, J. W. Amburgey, G. B. Cline, N. G. Anderson, L. L. McCauley, R. H. Stevens, and W. M. Swartout	Problems in biocontainment	Pp. 485–502 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Cline, G. B., C. E. Nunley, and N. G. Anderson	Improved continuous flow centrifugation with banding	*Nature* **212**, 487–89 (1966)
Congdon, C. C	Foreword and Comment on Cover	*Exptl. Hematol.* **9**, iii–vi (1966)
	Foreword and Comment on Cover	*Exptl. Hematol.* **10**, iii–vi (1966)
	Foreword and Comment on Cover	*Exptl. Hematol.* **11**, iii–vi (1966)
Congdon, C. C (editor)	Abstracts presented at the Bone Marrow Transplantation and Radiation Recovery Conference, held December 2 and 3, 1965, San Francisco, California (and two contributed papers)	*Exptl. Hematol.* **9**, Biology Division, Oak Ridge National Laboratory
	Abstracts presented at the Discussion Meeting on Bone Marrow Transplantation and Chemical Radiation Protection, held April 12, 1966, Atlantic City, New Jersey (and contributed paper)	*Exptl. Hematol.* **10**, Biology Division, Oak Ridge National Laboratory
	Some of the abstracts presented at the Conference on Germinal Centers of Lymphatic Tissue, held June 22–24, 1966, at the University of Bern, Bern, Switzerland (and two contributed papers)	*Exptl. Hematol.* **11**, Biology Division, Oak Ridge National Laboratory
Cook, J. S., and Jane K. Setlow	Photoreactivating enzyme in the sea urchin egg	*Biochem. Biophys. Res. Commun.* **24**, 285–89 (1966)
Davidson, Douglas	The onset of mitosis and DNA synthesis in roots of germinating beans	*Am. J. Botany* **53**, 491–95 (1966)
Day, J. W., and Rhoda F. Grell	Radiation-induced nondisjunction and loss of chromosomes in *Drosophila melanogaster* females II. Effects of exchange and structural heterozygosity	*Mutation Res.* **3**, 503–9 (1966)
de Serres, F. J.	Carbon dioxide stimulation of the *ad-3* mutants of *Neurospora crassa*	*Mutation Res.* **3**, 420–25 (1966)
Dolin, M. I.	NADH peroxidase	Pp. 171–82 in *Flavins and Flavoproteins, Proceedings of a Symposium Held in Amsterdam, The Netherlands, June 10–15, 1965*, ed. by E. C. Slater, Elsevier, Amsterdam, 1966
Ehling, U. H.	Dominant mutations affecting the skeleton in offspring of x-irradiated male mice	*Genetics* **54**, 1381–89 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Eisenberg, Sara, and Tuneo Yamada	A study of DNA synthesis during the transformation of the iris into lens in the lentectomized newt	*J. Exptl. Zool.* **162**, 353–67 (1966)
El-Aaser, A. A., E. Reid, E. Klucis, P. Alexander, J. T. Lett, and Jacqueline Smith	Resolution of the components in the microsomal fraction of liver in the B-IV zonal centrifuge	Pp. 323–32 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Fisher, W. D., and R. E. Canning	Isolation and characterization of rat macroglobulin	*Ibid.*, pp. 403–12
Foard, D. E., and A. H. Haber	Mitoses in thermodormant lettuce seeds with reference to histological location, localized expansion, and seed storage	*Planta* **71**, 160–70 (1966)
Foreman, C. W.	Inheritance of multiple hemoglobins in Peromyscus	*Genetics* **54**, 1007–12 (1966)
Goodman, Joan Wright, and H. B. Wheeler	Modification of poor growth of parental marrow cells in F_1 hybrid mice	*Nature* **211**, 1420–21 (1966)
Grant, W. C.	Effect of different fixatives on fine structure of the lymphocyte nucleus	*Exptl. Hematol.* **9**, 82–86 (1966)
Green, J. G.	Automated carbohydrate analyzer: Experimental prototype	Pp. 447–67 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Green, J. G., C. E. Nunley, and N. G. Anderson	High-pressure column chromatography. I. Design of apparatus and separation of bases, nucleosides, and nucleotides	*Ibid.*, pp. 431–40
Grell, Rhoda F.	The meiotic origin of temperature-induced crossovers in *Drosophila melanogaster* females	*Genetics* **54**, 411–21 (1966)
Grell, Rhoda F., E. R. Muñoz, and W. F. Kirschbaum	Radiation-induced nondisjunction and loss of chromosomes in *Drosophila melanogaster* females I. The effect of chromosome size	*Mutation Res.* **3**, 494–502 (1966)
Harris, W. W., N. G. Anderson, T. W. Bartlett, Elizabeth L. Rutenberg, L. L. McCauley, and R. M. Kniseley	Unusual particles in human plasma from leukemia and lymphosarcoma	Pp. 389–94 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Hastings, J. R. B., J. H. Parish, K. S. Kirby, and E. Klucis	The use of the zonal ultracentrifuge to separate components of ribosomal RNA	*Ibid.*, pp. 397–402
Hollaender, Alexander, S. F. Carson, and J. L. Liverman	Biology Division Semiannual Progress Report for Period Ending July 31, 1966	ORNL-3999
Kamrin, Michael	Changes in the capacity of algae to fluoresce during steady-state photosynthesis	*Biochim. Biophys. Acta* **126**, 262–268 (1966)
Kaufman, L. V., and K. Bruce Jacobson	Use of lactate dehydrogenase as a marker in rat-mouse chimera studies	*Cancer Res.* **26**, 1778–79 (1966)
Khym, J. X.	Partition of transfer ribonucleic acid between aqueous salt solutions and trichlorotrifluoroethane containing a quaternary amine	*J. Biol. Chem.* **241**, 4529–33 (1966)
Kilbey, B. J., and F. J. de Serres	Quantitative and qualitative aspects of photoreactivation of premutational ultraviolet damage at the *ad-3* loci of *Neurospora crassa*	*Mutation Res.* **4**, 21–29 (1967)
Kimball, R. F., and Stella W. Perdue	Comparison of mutagenesis by X-rays and triethylene melamine in *Paramecium*, with emphasis on the role of mitosis	*Mutation Res.* **4**, 37–50 (1967)
Kusama, Keiichi, D. M. Prescott, L. O. Fröholm, and W. E. Cohn	The formation and metabolic interchanges of pseudouridine in *Tetrahymena pyriformis*	*J. Biol. Chem.* **241**, 4086–91 (1966)
Lange, R. D., M. L. Simmons, and N. R. Dibelius	Polycythemic mice produced by hypoxia in silicone rubber membrane enclosures: A new technique	*Proc. Soc. Exptl. Biol. Med.* **122**, 761–64 (1966)
Leibo, S. P., and Peter Mazur	Effect of osmotic shock and low salt concentration on survival and density of bacteriophages T4B and $T4Bo_1$	*Biophys. J.* **6**, 747–72 (1966)
Makinodan, Takashi, and J. F. Albright	Proliferative and differentiative manifestations of cellular immune potential	Pp. 1–36 in *Progress in Allergy*, vol. 10 (ed. by Paul Kollós and B. H. Waksman), S. Karger, Basel/New York, 1967
Makinodan, Takashi, and W. J. Peterson	Further studies on the secondary antibody-forming potential of juvenile, young adult, adult, and aged mice	*Develop. Biol.* **14**, 112–29 (1966)
	Secondary antibody-forming potential of mice in relation to age – its significance to senescence	*Develop. Biol.* **14**, 96–111 (1966)
Malling, H. V.	Hydroxylamine as a mutagenic agent for *Neurospora crassa*	*Mutation Res.* **3**, 470–76 (1966)
	Methods for the identification of genetic alterations by specific revertibility tests in *Neurospora crassa*	*Neurospora Newsletter* **9**, 13–14 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Malling, H. V.	Mutagenicity of two potent carcinogens, dimethylnitrosamine and diethylnitrosamine, in *Neurospora crassa*	*Mutation Res.* **3**, 537–40 (1966)
Marin, Guglielmo, and M. A Bender	Radiation-induced mammalian cell death: Lapse-time cinemicrographic observations	*Exptl. Cell Res.* **43**, 413–23 (1966)
Mattingly, Ellen	Induction of chromosome- and chromatid-type aberrations by phleomycin	*Mutation Res.* **4**, 51–57 (1967)
	Synchrony of cell division in root meristems following treatment with 5-aminouracil	Pp. 256–68 in *Cell Synchrony: Studies in Biosynthetic Regulation*, ed. by I. L. Cameron and G. M. Padilla, Academic, New York and London, 1966
Mazur, Peter	Physical and chemical basis of injury in single-celled micro-organisms subjected to freezing and thawing	Pp. 213–315 in *Cryobiology*, ed. by H. T. Meryman, Academic, London and New York, 1966
McArthur, W. H.	Oxygen consumption in radiation chimeras	*Exptl. Hematol.* **11**, 47–51 (1966)
McGrath, R. A., and R. W. Williams	Reconstruction *in vivo* of irradiated *Escherichia coli* deoxyribonucleic acid; the rejoining of broken pieces	*Nature* **212**, 534–35 (1966)
Muhammed, Amir, and C. M. Steinberg	Action spectrum for cross-reactivation with various amber mutants of bacteriophage T4D	*J. Mol. Biol.* **21**, 395–97 (1966)
Nettesheim, Paul, Takashi Makinodan, and Carol J. Chadwick	Improved diffusion chamber cultures for cytokinetic analysis of antibody response	*Immunology* **11**, 427–39 (1966)
Odell, T. T., Jr., Marianne Jefferson, C. W. Jackson, and Rebecca S. Reiter	Effects of vinblastine on megakaryocytes and platelets of rats	*Exptl. Hematol.* **10**, 36–41 (1966)
Padilla, G. M., I. L. Cameron, and L. H. Elrod	The physiology of repetitively synchronized *Tetrahymena*	Pp. 269–88 in *Cell Synchrony: Studies in Biosynthetic Regulation*, ed. by I. L. Cameron and G. M. Padilla, Academic, New York and London, 1966
Popp, R. A.	Inheritance of an erythrocyte and kidney esterase in the mouse	*J. Heredity* **57**, 197–201 (1966)
Rahn, R. O.	Pyrimidine dimers: Effect of temperature on photoinduction	*Science* **154**, 503–4 (1966)
Randolph, M. L.	Medical and biological effects of ionizing radiations	*Nucl. Safety* **7**, 476–81 (1966)
Regan, J. D.	Alkaline phosphatase in synchronized human cells	*Experientia* **22**, 708–9 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Reimer, C. B., T. E. Newlin, M. L. Havens, R. S. Baker, N. G. Anderson, G. B. Cline, H. P. Barringer, and C. E. Nunley	An evaluation of the B-V (continuous-flow) and B-IV (density gradient) rotors by use of live polio virus	Pp. 375–88 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Roberts, P. A.	A tandem duplication that lowers recombination throughout a chromosome arm of *Drosophila melanogaster*	*Genetics* **54**, 969–79 (1966)
Seaborg, Glenn T.	Foreword	Pp. ix–x in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Setlow, Jane K.	Cytochrome b_2 is not photoreactivating enzyme	*Biochem. Biophys. Res. Commun.* **26**, 30–31 (1967)
	The molecular basis of biological effects of ultraviolet radiation and photoreactivation	Pp. 195–248 in *Current Topics in Radiation Research*, Vol. II, ed. by M. Ebert and A. Howard, North-Holland, Amsterdam, 1966
Seto, Frank	A correlated growth response of spleen and liver during the graft-versus-host reaction in the chick embryo	*Growth* **30**, 257–62 (1966)
Skinner, Dorothy M.	Breakdown and reformation of somatic muscle during the molt cycle of the land crab, *Gecarcinus lateralis*	*J. Exptl. Zool.* **163**, 115–23 (1966)
Smith, L. H., and Sarah D. Phillips	Relative erythropoietic competence of isologous bone marrow cells, spleen cells, peripheral leukocytes, and peritoneal fluid cells	*Exptl. Hematol.* **9**, 86–88 (1966)
Somers, W. R., R. A. Brown, and Takashi Makinodan	The possibility of determining the absolute number of hemagglutinating antibody molecules in antisera	*Immunochemistry* **3**, 343–45 (1966)
Stevens, R. H.	A flame ionization analyzer for the continuous, rapid determination of carbon in liquid streams or solid samples	Pp. 469–83 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Takata, Chinami, J. F. Albright, and Tuneo Yamada	Gamma crystallins in Wolffian lens regeneration demonstrated by immunofluorescence	*Develop. Biol.* **14**, 382–400 (1966)
Thomas, D. B., and C. C Congdon	Secondary atrophy in the regenerating thymus of the irradiated mouse	*Exptl. Hematol.* **11**, 45–47 (1966)
Tyndall, R. L., Ernestine Teeter, J. A. Otten, N. D. Bowles, J. G. Vidrine, A. C. Upton, and H. E. Walburg, Jr.	Further observations on *in vitro* cytopathic effects associated with murine leukemia virus infection	*Intern. J. Cancer* **1**, 565–72 (1966)
Upton, A. C., V. K. Jenkins, H. E. Walburg, Jr., Niel Wald, R. L. Tyndall, and J. W. Conklin	Observations on viral, chemical, and radiation-induced myeloid and lymphoid leukemias in RF mice	Pp. 329–47 in *Conference on Murine Leukemia* (ed. by M. A. Rich and J. B. Moloney), *Natl. Cancer Inst. Monograph* **22**, U. S. Department of Health, Education, and Welfare; Public Health Service, Bethesda, Maryland, 1966
Uziel, Mayo	Stability of 4,4′-di(thiouridine) and 4-thiouridine in alkali	*Biochem. Biophys. Res. Commun.* **25**, 105–8 (1966)
von Borstel, R. C., O. L. Miller, Jr., and Romance F. Carrier	X-radiation-induced breakage of lampbrush chromosomes	Pp. 141–44 in *Chromosomes Today*, Vol. 1, ed. by C. D. Darlington and K. R. Lewis, Oliver & Boyd, Edinburgh, 1966
Wallace, R. A., D. W. Jared, and A. Z. Eisen	A general method for the isolation and purification of phosvitin from vertebrate eggs	*Can. J. Biochem.* **44**, 1647–55 (1966)
Whiting, P. W.	"Black" eye colors of *Mormoniella*	*Genetics* **54**, 639–55 (1966)
Whitson, G. L., G. M. Padilla, R. E. Canning, I. L. Cameron, N. G. Anderson, and L. H. Elrod	The isolation of oral structures from *Tetrahymena pyriformis* by low-speed zonal centrifugation	Pp. 317–21 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Whitson, G. L., G. M. Padilla, and W. D. Fisher	Morphogenic and macromolecular aspects of synchronized *Tetrahymena*	Pp. 289–306 in *Cell Synchrony: Studies in Biosynthetic Regulation*, ed. by I. L. Cameron and G. M. Padilla, Academic, New York and London, 1966
Williams, D. B.	Effects of x rays on cell lethality and micronuclear number in the ciliate *Spathidium spathula*	*J. Protozool.* **13**, 272–77 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Wright, R. R., W. S. Pappas, J. A. Carter, and C. W. Weber	Preparation and recovery of cesium compounds for density gradient solutions	Pp. 241–49 in *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis* (ed. by N. G. Anderson), *Natl. Cancer Inst. Monograph* **21**, U. S. Department of Health, Education, and Welfare; Public Health Service, Baltimore, Maryland, 1966
Yamamoto, Hiroshi	Reversible transformation of lymphocytes in human leukocyte cultures	*Nature* **212**, 997–98 (1966)

LECTURES

During the period August 1, 1966, to January 31, 1967, members of the Biology Division presented 206 lectures and speeches. Of these, 91 were given at professional society meetings; 82 at the invitation of universities, laboratories, scientific institutions, and professional groups or clubs in the United States and abroad; and 33 under the Traveling Lecture Program.

For details on speaking activities 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.	Genetic control of cell division in bacteria	University of Georgia, Athens
	The genetic control of radiation sensitivity	(1) Emory University, Atlanta, Georgia (2) Tuskegee Institute, Tuskegee Institute, Alabama (3) Northwestern State College of Louisiana, Natchitoches
	Genetic control of radiation sensitivity in bacteria	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tennessee (2 lectures)
	The repair of radiation damage to the cell division mechanism of bacteria	(1) University of Texas, Austin (2) Texas Woman's College, Denton (3) Kansas State University, Manhattan (4) University of Missouri Medical School, Columbia (5) Louisiana State University, Baton Rouge

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I. (W. D. Fisher and G. E. Stapleton)	Genetic control of cell division in bacteria [*Science* **154**, 417 (1966)]	1966 Autumn Meeting of the National Academy of Sciences, Duke University, Durham, North Carolina
Albright, J. F.	Immunology and scientific research	16th Nuclear Science Seminar, ORAU, Oak Ridge, Tennessee
Allen, R. C.	Esterase changes in hypoxia and their relationship to erythropoietin production	Tennessee Blood Society Meeting, Gatlinburg, Tennessee
Anderson, N. G.	Biophysical separations techniques	Workshop NIH Pathology "B" Study Section, Oak Ridge, Tennessee
	The Molecular Anatomy Program	(1) Oak Ridge Section, Institute of Electrical and Electronic Engineers, Oak Ridge, Tennessee (2) Student Trainees and Graduate Fellows, ORAU, Oak Ridge, Tennessee
	Separation systems for the Molecular Anatomy Program	Eli Lilly Lecture, 26th Series, Eli Lilly and Company, Indianapolis, Indiana
Arnold, W. A.	Molecular and genetical biology	University of Utah, Salt Lake City
Axelrod, D. E.	(1) DNA and the basis of biochemical genetics (2) The expression of mutations	University of Tennessee, Knoxville
Axelrod, D. E. (and H. I. Adler)	A correlation between increased radiation resistance and increased cell size of *Escherichia coli*	Am. Soc. Microbiol., Kentucky-Tennessee Branch, Nashville, Tennessee
	A radiation-resistant mutant of *Escherichia coli* K-12 [*Genetics* **54**, 318 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
Ball, Frances L. (W. W. Harris, Frances L. Ball, Helen Coates, G. B. Cline, N. G. Anderson, and R. M. Chanock)	Ultrastructure of PPLO free respiratory syncytial virus (Long strain)	Electron Microscopy Society of America, San Francisco, California

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Barnett, W. E.	Mitochondrial transfer RNAs	Thomas Hunt Morgan Centennial Symposium, Lexington, Kentucky
	Multiple aminoacyl-RNA synthetase systems	(1) University of South Florida, Tampa (2) Florida Presbyterian College, St. Petersburg
	The roles of RNA in protein synthesis	Randolph-Macon Woman's College, Lynchburg, Virginia
Barnett, W. E. (and D. H. Brown)	Evidence for the association of a phenylalanine tRNA with Neurospora mitochondria [*Science* **154**, 417 (1966)]	1966 Autumn Meeting of the National Academy of Sciences, Duke University, Durham, North Carolina
Bender, M. A (and M. A. Kastenbaum)	Human chromosome measurement	Conference on Mammalian Cytology and Somatic Cell Genetics, Saint-Simons Island, Georgia
Bertsch, W. F.	Photosynthesis	Yale University, New Haven, Connecticut
	Solid state phenomena in photosynthesis	Biology Colloquium, Harvard University, Cambridge, Massachusetts
Brewen, J. G.	Sister chromatic exchanges: are they spontaneous or induced?	ANZAAS – Genetical Society Cytogenetics Symposium, Melbourne, Australia
Brick, J. O. (L. J. Serrano and N. L. Ensor)	Variation in breeding production among colonies of RFM mice under conditions of germfree, barrier, SPF, and conventional housing [Abstr. of papers, 17th Annual Meeting, Animal Care Panel, 1966, p. 92]	Animal Care Panel, 17th Annual Meeting, Chicago, Illinois
Brown, R. C. (and B. H. Smith)	Malacoplakia of the testis	College of American Pathologists and American Society of Clinical Pathologists, Annual Meeting, Washington, D.C.
Caro, L. G.	The attachment of the DNA bacteriophage f1 to male strains of *E. coli*	European Phage Meeting, Naples, Italy
	The attachment of f1 bacteriophage to male strains of *Escherichia coli*	Second International Congress of Biophysics, Vienna, Austria

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Cohn, W. E.	Biochemical nomenclature	Symposium on Chemical Nomenclature, Am. Chem. Soc., 152nd National Meeting, New York City
	Chromatography of nucleic acid components (2 lectures)	Course on Nucleic Acids, Rockefeller University, New York City
	Some recent developments in chromatography	Rockefeller University, New York City
Congdon, C. C	Bone marrow transplantation	Blount Memorial Hospital, Maryville, Tennessee
	Pathological processes	University of Tennessee, Knoxville
Congdon, C. C (M. G. Hanna, Jr., and M. L. Simmons)	The effect of a viral agent on lymphatic tissues [*Exptl. Hematol.* **12**, 21–22 (1967)]	Bone Marrow Conference, University of Michigan, Ann Arbor
Congdon, C. C (Mary Sue Wedemeyer, and M. L. Simmons)	Initiation of immune response in mice subject to hypoxia [*Exptl. Hematol.* **12**, 92–94 (1967)]	Bone Marrow Conference, University of Michigan, Ann Arbor
Congdon, C. C (and D. G. Doherty)	The effect of bone marrow dose on the LD_{50} response of x-irradiated mice [*Exptl. Hematol.* **12**, 75–76 (1967)]	Bone Marrow Conference, University of Michigan, Ann Arbor
Cook, Dorothy S.	The lifetime of ribosomal RNA in nongrowing epidermis	Second International Congress of Biophysics, Vienna, Austria
Cook, J. S.	Non-solvent water in human erythrocytes	Second International Congress of Biophysics, Vienna, Austria
Corbett, J. R.	Do lysosomes exist in plants? [*Plant Physiol.*, Proceedings Issue, lxxiv–lxxv (1966)]	AIBS (Am. Soc. Plant Physiol.), College Park, Maryland
	Purification of lysosomes by zonal centrifugation	Meeting No. 467, Biochemical Society, Cardiff, Wales
Cosgrove, G. E.	Delayed somatic effects of radiation	Ten-Week Health Physics Course, ORAU, Oak Ridge, Tennessee
	Parasites as an oncogenic factor in wildlife [*Bull. Wildlife Disease Assoc.* **2**, 67 (1966)]	AIBS (Wildlife Disease Assoc.), College Park, Maryland
	Pathology of parasitism in wildlife	Virginia Institute of Marine Science, Gloucester Point, Virginia

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Cosgrove, G. E. (H. E. Walburg, Jr., and A. C. Upton)	Gastrointestinal lesions in aged conventional and germfree mice exposed to radiation as young adults	Symposium on Gastrointestinal Radiation Injury, Richland, Washington
Curtiss, Roy, III	Mechanisms of chromosome mobilization and transfer during bacterial conjugation	Thomas Hunt Morgan Centennial Symposium, Lexington, Kentucky
	Mechanisms involved during bacterial conjugation in *Escherichia coli* K-12	Washington University School of Medicine, St. Louis, Missouri
	(1) Quantitative analysis in virology (2) Radiobiology of viruses	University of Tennessee, Knoxville
Curtiss, Roy, III (and L. J. Charamella)	Role of the F^- parent during bacterial conjugation in *Escherichia coli* [*Genetics* **54**, 329–30 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
Curtiss, Roy, III (D. R. Stallions, J. A. Mays, and Janet Renshaw)	Mechanism of chromosome mobilization and transfer by F^+ donors of *Escherichia coli* K-12	Am. Soc. Microbiol., Kentucky-Tennessee Branch, Nashville, Tennessee
Doherty, D. G.	An experimental approach to inhalation carcinogenesis	Conference on Environmental Control in Hospitals, American Hospital Association, Chicago, Illinois
Donnellan, J. E., Jr.	Effects of radiation during spore germination	Institut Pasteur, Paris, France
	Recovery of spores and vegetative cells from radiation damage	Medical-Biological Laboratory RVO-TNO, Rijswijk, The Netherlands
Donnellan, J. E., Jr. (and R. S. Stafford)	Effect of UV irradiation on spore DNA	Second International Congress of Biophysics, Vienna, Austria
Dumont, J. N.	The response of muscle cells to invasion by *Trichinella spiralis* [*J. Cell Biol.* **31**, 30A (1966)]	Am. Soc. Cell Biol., 6th Annual Meeting, Houston, Texas
Ehling, U. H.	Genetic control of radiation sterilization of female mice [*Genetics* **54**, 331–32 (1966)] (read by title)	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
	Radiogenetics	AEC-NSF Basic Institute for Nuclear Science and Engineering, Oak Ridge, Tennessee

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Elrod, L. H. (and N. G. Anderson)	Isolation of nuclei in sucrose-D_2O gradients	Tennessee Academy of Science, 76th Annual Meeting, East Tennessee State University, Johnson City
Fisher, W. D.	(1) Cell fraction (2) Cell division in *E. coli*	Syracuse University, Syracuse, New York
Foard, D. E.	Studies of thermodormant lettuce seed	University of Tennessee, Knoxville
Foard, D. E. (and A. H. Haber)	Mitotic activity in thermodormant lettuce seeds with reference to histological location and localized expansion [*Plant Physiol.*, Proceedings Issue, xliii (1966)]	AIBS (Am. Soc. Plant Physiol.), College Park, Maryland
Goodman, Joan Wright (and H. B. Wheeler)	Improvement of poor growth of parental hemopoietic cells	XI Congress of the International Society of Blood Transfusion, Sydney, Australia
Green, J. G.	The AEC-NIH MAN program at the Biology Division, ORNL	College of Veterinary Medicine, Iowa State University, Ames
	Biologists and Baptists: Conflict or complement	Carson-Newman College, Jefferson City, Tennessee
	The Oak Ridge Molecular Anatomy Program: Concept and achievement	(1) Research Society of America, Bound Brook, New Jersey (2) University of Kentucky, Lexington
Grell, E. H. (K. B. Jacobson and J. B. Murphy)	Genetic analysis of alcohol dehydrogenase isozymes in Drosophila	New York Academy of Sciences Conference on Multiple Forms of Enzymes, New York City
Grell, Rhoda F.	Homologous and nonhomologous recognition in *Drosophila melanogaster*	Symposium on Mutual Recognition Between Genetic Material, Genetics Society of America, Chicago, Illinois
Gude, W. D.	Autoradiography	ORAU Medical Radioisotopes Course, Oak Ridge, Tennessee
Haber, A. H.	Experimental analysis of plant development	West Virginia University, Morgantown

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Haber, A. H. (and D. E. Foard)	Experimental separations of growth, differentiation, and cell division in intact plants	AIBS Symposium: Are Growth and Differentiation Incompatible? (cosponsored by the Developmental Biology Division of the Am. Soc. Plant Physiol.), College Park, Maryland
Haber, A. H. (and Patricia L. Walne)	Actions of gamma radiation on senescence and death in excised wheat leaf tissue after normal cessation of cell growth and division	64th Annual Convention of the Southern Agricultural Workers, Inc., New Orleans, Louisiana
Hanna, M. G., Jr.	Morphologic changes in spleen lymphatic tissue during antibody production	(1) National Cancer Institute, Bethesda, Maryland (2) Louisiana State University, Baton Rouge
Hanna, M. G., Jr. (and Paul Nettesheim)	Studies of lymphatic tissue germinal centers during an immune reaction: Localization of ^{125}I-labeled heterologous and isologous proteins [*RES: J. Reticuloendothelial Soc.* **3**, 363 (1966)]	Reticuloendothelial Society, 3rd Annual Meeting, Bethesda, Maryland
Harrison, A. P., Jr.	Susceptibility of *Escherichia coli* to photodynamic action by benzo(a)pyrene [*J. Cell Biol.* **31**, 45A (1966)]	Am. Soc. Cell Biol., 6th Annual Meeting, Houston, Texas
Hollaender, Alexander	Closing remarks	International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico
Jacobson, K. Bruce	Transfer RNA: Interaction with synthetases and codons	(1) St. Bonaventure University, Olean, New York (2) Rosary Hill College, Buffalo, New York (3) University of North Dakota, Grand Forks (4) University of Minnesota, Minneapolis
	Transfer RNA: Structure and function	Marquette University, Milwaukee, Wisconsin
Jamison, R. M.	Studies on papovavirus SV40 tumor antigen	University of Southwestern Louisiana, Lafayette
Jones, R. T.	Environmental monitoring using germfree mice	Am. Soc. Microbiol., Kentucky-Tennessee Branch, Nashville, Tennessee

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Kenney, F. T. (D. D. Holten and W. L. Albritton)	Hormonal regulation of liver enzymes	International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico
Khym, J. X.	Partition of transfer ribonucleic acid between aqueous salt solutions and trichlorotrifluoroethane containing a quaternary amine [Abstr. of papers, 152nd Meeting, Am. Chem. Soc., 1966, p. 68C]	Am. Chem. Soc., 152nd National Meeting, New York City
Kimball, R. F.	Comparison of repair and fixation processes with radiation and chemical mutagens [*Science* **154**, 422 (1966)]	1966 Autumn Meeting of the National Academy of Sciences, Duke University, Durham, North Carolina
	Variation in DNA content and its effect on cell growth and division in cell lineages in *Paramecium aurelia* [*J. Cell Biol.* **31**, 60A (1966)]	Am. Soc. Cell Biol., 6th Annual Meeting, Houston, Texas
Leibo, S. P.	Biology of bacteriophage	Washington and Jefferson College, Washington, Pennsylvania
	Freezing of biological systems	(1) Virginia Military Institute, Lexington (2) Washington and Jefferson College, Washington, Pennsylvania
	Freezing damage in bacteriophage T4	University of Texas, Austin
Lindsley, D. L.	The recovery of mutants affecting meiosis from natural populations of Drosophila	(1) Institute of Zoology of the University of Munich, Munich, Germany (2) Max Planck Institute for Biology, Tubingen, Germany (3) Institute of Radiation Genetics, University of Leiden, Leiden, The Netherlands (4) University of California at San Diego, La Jolla
Longworth, J. W.	Luminescence and ESR of poly-L-tyrosine and related compounds	Wake Forest College, Winston-Salem, North Carolina
	Luminescence of nucleic acids and proteins	Radiation Research Laboratory, University of Notre Dame, Notre Dame, Indiana

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Longworth, J. W. (and R. O. Rahn)	Luminescence and E.S.R. of nucleic acid protein	Institute for Macromolecular Research, CNRS, Strasbourg, France
	Photoinduced luminescence and triplet ESR of poly-L-tyrosine	Second International Congress of Biophysics, Vienna, Austria
Makinodan, Takashi (Paul Nettesheim, Toshiteru Morita, and Carol J. Chadwick)	Antibody synthesis several days after exposure of functional spleen cells to 10,000 r [*Science* **154**, 423 (1966)]	1966 Autumn Meeting of the National Academy of Sciences, Duke University, Durham, North Carolina
Mattingly, Ellen	Variations in metabolic activity during the larval development of Rhynchosciara [*J. Cell Biol.* **31**, 74A (1966)]	Am. Soc. Cell Biol., 6th Annual Meeting, Houston, Texas
Mazur, Peter	Causes of injury from intracellular freezing	Conference on Basic Mechanisms of Freezing Injury, Fairbanks, Alaska
	Mechanism of injury in cells during freezing and thawing	CSIRO, Division of Food Preservation, Sydney, Australia
	Physical-chemical basis of freezing injury in living cells	University of Melbourne, Melbourne, Australia
	Physical-chemical basis of injury from intracellular freezing in yeast	International Conference on Cryobiology, Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan
	Physical and chemical changes during freezing and thawing of cells	Symposium on New Methods of Preservation of Blood and Bone Marrow, XI Congress of the International Society of Blood Transfusion, Sydney, Australia
McDevitt, D. S.	Separation and characterization of the lens proteins of the amphibian *Rana pipiens* [*Am. Zool.* **6**, 335–36 (1966)]	AIBS (Am. Soc. Zool.), College Park, Maryland
McEwen, W. S.	Opportunities for Fisk graduates at Oak Ridge National Laboratory	Fisk University, Nashville, Tennessee
McGrath, R. A.	Cellular repair of irradiated DNA	Bowman Gray School of Medicine, Winston-Salem, North Carolina
	DNA repair	Lawrence Radiation Laboratory, Livermore, California
Montour, J. L.	Irradiation as a stress: Effects on the lymphatic system	University of Tennessee, Knoxville

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Muhammed, Amir	Reactivation phenomena in uv-irradiated phage	Southwest Center for Advanced Studies, Dallas, Texas
Nettesheim, Paul (and M. G. Hanna, Jr.)	Recovery potential of uncommitted and committed immunological competent cells from x-ray insults [*RES: J. Reticuloendothelial Soc.* **3**, 362 (1966)]	Reticuloendothelial Society, 3rd Annual Meeting, Bethesda, Maryland
Novelli, G. David	(Invited discussant)	International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico
	Protein synthesis	Third Annual Pathobiology Meeting, Aspen, Colorado
	Some general aspects of protein biosynthesis	Midwinter Conference of Immunologists, Honolulu, Hawaii
Popp, R. A.	Esterase variants in mice	University of Michigan, Ann Arbor
	Growth of bone marrow in syngeneic, F_1, and allogeneic recipients [*Exptl. Hematol.* **12**, 74 (1967)]	Bone Marrow Conference, University of Michigan, Ann Arbor
Prestayko, A. W. (and W. D. Fisher)	Isolation and characterization of ribosomes from *Tetrahymena pyriformis* [*J. Cell Biol.* **31**, 88A (1966)]	Am. Soc. Cell Biol., 6th Annual Meeting, Houston, Texas
Rahn, R. O.	The influence of conformation on the photochemistry of nucleic acids	Brandeis University, Waltham, Massachusetts
Rahn, R. O. (and J. W. Longworth)	Excited state properties of polynucleotides at 77°K	Institute for Macromolecular Research, CNRS, Strasbourg, France
	Pyrimidine dimers: Effect of temperature on photoinduction	Second International Congress of Biophysics, Vienna, Austria
Regan, J. D.	Aspects of the response of human cells *in vitro* to x and uv radiation	Florida Atlantic University, Boca Raton, Florida
	The fate of uv-induced pyrimidine dimers in human cells *in vitro*	National Institute of Arthritis and Metabolic Diseases, NIH, Bethesda, Maryland
	Studies of homeotherm and poikilotherm cells *in vitro*	Institute of Marine Science, University of Miami, Miami, Florida

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Oakberg, E. F.	Effects of ionizing radiation on the testis of man	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tennessee
	^{3}H-Uridine labeling of mouse oocytes	Colloquium on Physiology and Reproduction in Mammals, Paris, France
Odell, T. T., Jr. (and C. W. Jackson)	How do megakaryocytes differentiate?	XI Congress of the International Society of Haematology, Sydney, Australia
Papaconstantinou, John	An analysis of the ribosomal breakdown in lens fiber cell differentiation	Symposium on the Biochemistry of the Eye, Tutzing, Germany
	Molecular aspects of lens cell differentiation	Duke University Medical Center, Durham, North Carolina
Papaconstantinou, John (Emilia Julku and Dorothy M. Schenck)	Nucleic acid changes associated with lens cell differentiation	Second International Biophysics Congress, Vienna, Austria
Perkins, E. H. (Paul Nettesheim and Toshiteru Morita)	Radioresistance of the engulfing and degradative capacities of peritoneal phagocytes to kiloroentgen x-ray doses	Institut Ruder Boškovic, Zagreb, Yugoslavia
Perkins, E. H. (Paul Nettesheim, Toshiteru Morita, and H. E. Walburg, Jr.)	The engulfing potential of peritoneal phagocytes from conventional germfree mice	International Symposium on Atherosclerosis and the Reticuloendothelial System, Como, Italy
Perondini, A. L. P. (Crodowaldo Pavan and A. L. P. Perondini)	Changes in chromosomes induced by infection in cells of *Sciara* [*Genetics* **54**, 353 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
Roberts, P. A. (R. F. Kimball and Crodowaldo Pavan)	Response of Rhynchosciara chromosomes to microsporidian infection: Increased polyteny and generalized puffing [*Genetics* **54**, 357 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
Robie, D. M. (M. L. Simmons and L. J. Serrano)	The effects of a filter cover on temperature and humidity in a mouse cage [Abstr. of papers, 17th Annual Meeting, Animal Care Panel, 1966, p. 12]	Animal Care Panel, 17th Annual Meeting, Chicago, Illinois
Romani, R. J.	Recovery from intracellular radiation damage as a function of cell age	Gerontological Society, 19th Annual Meeting, New York City
Russell, Liane B.	The relative sensitivity of various germ-cell stages of the mouse to radiation-induced sex-chromosome abnormalities	(1) Puerto Rico Nuclear Center, Mayaguez (2) Puerto Rico Nuclear Center, Rio Piedras

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Russell, Liane B. (and C. Saylors Montgomery)	Radiation-induced sex-chromosome abnormalities in female germ-cell stages of the mouse [*Genetics* **54**, 358 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
Russell, W. L.	Radiation biology — genetic effects	Ten-Week Health Physics Course, ORAU, Oak Ridge, Tennessee
	Recent advances in mammalian radiation genetics	(1) Puerto Rico Nuclear Center, Mayaguez (2) Puerto Rico Nuclear Center, Rio Piedras
	Recent findings in mammalian radiation genetics and their application to health physics	New England Chapter of the Health Physics Society, Cambridge, Massachusetts
Russell, W. L. (and Elizabeth M. Kelly)	Mutation frequency in female mice exposed to high intensity x irradiation delivered in small fractions [*Science* **154**, 427–28 (1966)]	1966 Autumn Meeting of the National Academy of Sciences, Duke University, Durham, North Carolina
Serrano, L. J. (and Carlene Amsbury)	Semiautomatic recording and computer processing of mouse breeding data [Abstr. of papers, 17th Annual Meeting, Animal Care Panel, 1966, p. 93]	Animal Care Panel, 17th Annual Meeting, Chicago, Illinois
Serrano, L. J. (E. B. Darden, Jr., L. J. Serrano, and L. W. Harris, Jr.)	A subcutaneous implant for telemetering body temperature of small mammals [Abstr. of papers, 17th Annual Meeting, Animal Care Panel, 1966, p. 95]	Animal Care Panel, 17th Annual Meeting, Chicago, Illinois
Setlow, R. B.	Photochemistry and photobiology of polynucleotides	Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois
Simmons, M. L.	(1) Care and housing of laboratory animals (2) Establishing an animal testing program and diagnostic service laboratory	University of California at Davis
	(1) Diseases of guinea pigs and hamsters (2) Operation of a laboratory animal facility	Washington State University, Pullman
	The proper care and housing of laboratory animals	Shepherd College, Shepherdstown, West Virginia
	Review of some latent murine virus diseases and establishing a virus testing program	University of Tennessee, Knoxville
Simmons, M. L. (and John Franklin)	Acute radiation mortality in mice inoculated with pigmented or nonpigmented *Pseudomonas* [Abstr. of papers, 17th Annual Meeting, Animal Care Panel, 1966, p. 1]	Animal Care Panel, 17th Annual Meeting, Chicago, Illinois

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Simões, L. C. G. (and Crodowaldo Pavan)	Chromosome activity in salivary gland of *Rhynchosciara* maintained *in vitro* [*Genetics* **54**, 362 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
Smith, R. H. (and Anna R. Whiting)	X-radiation sensitivity of *Habrobracon* oocytes at diakinesis [*Genetics* **54**, 364 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois
Steinberg, C. M.	The mechanism of inactivation of bacteriophage T4 by incorporated radioactive isotopes	University of Oregon, Eugene
Steinberg, C. M. (and R. C. von Borstel	Mechanism of killing by decay of radioactive phosphorus in bacteriophage T4 [*Science* **154**, 429 (1966)]	1966 Autumn Meeting of the National Academy of Sciences, Duke University, Durham, North Carolina
Stevens, Audrey L. (Nancy Sternberger and A. J. Emery, Jr.)	Studies of *E. coli* RNA polymerase and its complexes with λ DNA [Abstr. of papers, 152nd Meeting, Am. Chem. Soc., 1966, p. 68C]	Am. Chem. Soc., 152nd National Meeting, New York City
Stine, G. J.	Nicotinamide-adenine dinucleotidase activity during germination and growth of *Neurospora crassa*	Am. Soc. Microbiol., Kentucky-Tennessee Branch, Nashville, Tennessee
Stulberg, M. P.	(Invited discussant)	International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico
Terry, C. E. (and Jane K. Setlow)	Photoreactivating enzyme in Neurospora	Am. Soc. Microbiol., Kentucky-Tennessee Branch, Nashville, Tennessee
Upton, A. C.	Design of radiobiologic experiments	Workshop NIH Pathology "B" Study Section, Oak Ridge, Tennessee
	The pathology of ionizing radiation	University of Chicago Medical School, Chicago, Illinois
	The problem of cancer	Science Club, Webb School, Knoxville, Tennessee
	Radio-somatics	AEC-NSF Basic Institute for Nuclear Science and Engineering, Oak Ridge, Tennessee
Van Pelt, Gladys S.	The problem of gene dosage in *Habrobracon* [*Genetics* **54**, 367–68 (1966)]	Genetics Society of America, 35th Annual Meeting, Chicago, Illinois

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Volkin, Elliot	Enzyme induction by bacteriophage	International Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mexico
von Borstel, R. C.	The Habrobracon experiment in the Biosatellite Project	Hangar S, Cape Kennedy, Florida
von Borstel, R. C. (P. D. Panum and C. M. Steinberg)	Nature of the tr_{1-1} super-suppressors in Saccharomyces [*Science* **154**, 430 (1966)]	1966 Autumn Meeting of the National Academy of Sciences, Duke University, Durham, North Carolina
Walburg, H. E., Jr.	Aging in irradiated and unirradiated germfree mice	Roswell Park Memorial Institute, Buffalo, New York
Wallace, R. A.	Vitellogenesis in vertebrates	University of Michigan, Ann Arbor
Wei, C. H. (and L. F. Dahl)	Structure of and bonding in two tetrameric cobalt carbonyl sulfur complexes	American Crystallographic Association, Atlanta, Georgia
Weisberg, R. A.	Studies on temperature-sensitive cI mutants of bacteriophage lambda	(1) Yale University, New Haven, Connecticut (2) Harvard Medical School, Boston, Massachusetts
Whiting, Anna R.	Factors influencing the radiosensitivity of cells	AAAS (Sigma Delta Epsilon Meeting), Washington, D.C.
	Radiation biology and genetics of two hymenopteran species	Conference on Radiation Biology (sponsored by the U.S. Dept. of Agriculture), Fargo, North Dakota
Whitson, G. L.	Macromolecular events in synchronized cells	University of Georgia, Athens
Whitson, G. L. (and J. G. Green)	Changes in soluble carbohydrates of synchronized *Tetrahymena* [*J. Cell Biol.* **31**, 123A (1966)]	Am. Soc. Cell Biol., 6th Annual Meeting, Houston, Texas
Wicks, W. D.	Control of enzyme activity during development	(1) Virginia Polytechnic Institute, Blacksburg, Virginia (2) University of Alabama Medical Center, Birmingham
	Hormones: Biochemical regulators	(1) Marywood College, Scranton, Pennsylvania (2) Maryville College, Maryville, Tennessee (3) University of Richmond, Richmond, Virginia

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Wicks, W. D.	RNA synthesis and enzyme induction in fetal rat liver	Georgetown University, Washington, D.C.
	RNA synthesis in fetal rat liver	(1) Bowman Gray School of Medicine, Winston-Salem, North Carolina (2) University of Alabama Medical Center, Birmingham
Wust, C. J.	Biochemical studies on antibody synthesis	(1) Briar Cliff College, Sioux City, Iowa (2) Morningside College, Sioux City, Iowa (3) Westmar College, LeMars, Iowa
Yamada, Tuneo	Differentiation of lens cells	Conference on Morphological and Biochemical Aspects of Cytodifferentiation, Valbella, Switzerland
Zapisek, W. F.	Ribosomal breakdown during chick lens development	Los Alamos Scientific Laboratory, Los Alamos, New Mexico
Zimmerman, B. K.	The mechanism of chain initiation in the synthesis of polynucleotides by *M. lysodeikticus* DNA polymerase	Second International Congress of Biophysics, Vienna, Austria

VISITING LECTURERS

During the period August 1, 1966 – January 31, 1967, 46 lectures were given on the Biology Division Seminar Program by guest speakers from scientific institutions and universities in this country and abroad. Visiting lecturers from foreign countries included scientists from Australia, Belgium, Brazil, Canada, England, Germany, Italy, The Netherlands, Peru, Poland, Sweden, and Venezuela.

SPEAKER	AFFILIATION	SUBJECT
K. C. Atwood	Department of Microbiology, University of Illinois, Urbana	Genetics of the *Ur*-gene
Henry Baker	Johns Hopkins Medical Institute, Primate Center, Baltimore, Maryland	Latent haemobartonella infections in laboratory rodents
Hans J. Becker	Universität München, München, Germany	X-ray-induced somatic crossing over

SPEAKER	AFFILIATION	SUBJECT
Robert W. Bernlohr	Department of Microbiology, University of Minnesota Medical School, Minneapolis	Control of several metabolic pathways during intracellular development in Bacillus
E. Boyland	Chester Beatty Research Institute, London, England	The biochemistry of carcinogenesis
Reinier Braams	Department of Radiobiophysics, Fysisch Laboratorium, Rijksuniversiteit, Utrecht, The Netherlands	Radiation effects in proteins
R. D. Brock	Division of Plant Industry, CSIRO, Canberra City, Australia	Mutation-induced quantitative variation in plants
Arthur Brown	Virus Research Division, Fort Detrick, Frederick, Maryland	Conditional lethal mutants of animal viruses
Richard Carp	Wistar Institute, Philadelphia, Pennsylvania	The induction of two virus specific proteins by the tumor producing virus, SV_{40}
Kevin J. Crowley	Instituto Venezolano de Científicas Investigaciones, Caracas, Venezuela	Some olefin photoisomerizations
B. Zane Egan	Chemical Technology Division, Oak Ridge National Laboratory Oak Ridge, Tennessee	Measurements of the molecular weight of tRNAs by gel permeation chromatography and membrane osmometry
Jacques R. Fresco	Department of Chemistry, Princeton University, Princeton, New Jersey	Tertiary structure of transfer RNA
Earl Frieden	Florida State University, Tallahassee	Thyroid hormones and the biochemistry of amphibian metamorphosis
Jon Gallant	Department of Genetics, University of Washington, Seattle	Control of RNA synthesis in *E. coli*
Jerome J. Gambino	Bioastronautics Laboratory, Northrop Space Laboratories, Hawthorne, California	The relationship between cell population kinetics and radiation resistance in pocket mice
Giovanni Giudice	Istituto di Anatomia Comparata, Palermo, Italy	Ribosomes in sea urchin embryos
E. Goldberg	Tufts University Medical School, Boston, Massachusetts	Measurement of the number of nucleotide pairs in a genetic interval of phage T4
Walter R. Guild	Duke University, Durham, North Carolina	Transformation and transcription properties of fractionated DNA strands

SPEAKER	AFFILIATION	SUBJECT
R. D. Heidenreich	Bell Telephone Laboratories, Murray Hill, New Jersey	Phase contrast and high resolution
Philip E. Hildreth	Department of Zoology, University of California, Berkeley	Conception and misconception: embryonic development in *Drosophila melanogaster*
Ben Hochman	Department of Zoology, The University of Tennessee, Knoxville	Lethal loci on chromosome 4 of *D. melanogaster*
James W. Holleman	Department of Chemistry, Northwestern University, Evanston, Illinois	Primary structure of the non-heme respiratory protein hemerythrin
Carroll K. Johnson	Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee	Stereoscopic drawings illustrating the three-dimensional structure of the protein myoglobin
Edward Kowalski	Institute of Hematology, Warsaw, Poland	Defibrination syndrome
L. Leifer	Michigan Technological University, Houghton	Some implications of the Mössbauer effect for biochemistry
John Love	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tennessee	Radiation-induced changes in the synthesis of anthocyanin pigments in higher plants
J. R. Maisin	Radiobiology Department, Laboratories du C.E.N. à Mol-Donk, Mol-Donk, Belgium	Recent observations on chemical protection
Peter Moens	Department of Biology, York University, Toronto, Ontario, Canada	Chromosome behaviour at meiotic prophase
Pablo Mori-Chavez	Laboratorio de Investigacion de Cancer, Lima, Peru	Comments on Ninth International Cancer Congress, Tokyo, Japan, October 23–28, 1966
Montrose J. Moses	Duke University School of Medicine, Durham, North Carolina	DNA, chromosome differentiation and motility in an aflagellate sperm of a coccid
Erling Norrby	Department of Virus Research, Karolinska Institutet, Stockholm, Sweden	Soluble components of adenoviruses
Donald E. Olins	Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire	Model nucleoprotein complexes
Ellen Rasch	Marquette University, Milwaukee, Wisconsin	Microspectrophotometric measurements of polytene chromosomes

SPEAKER	AFFILIATION	SUBJECT
Gunter Röhrborn	University of Heidelberg, Heidelberg, Germany	Investigations with chemical mutagens in mammals
Roger Romani	University of California, Davis	Radiation effects on plant mitochondria
Royal F. Ruth	Department of Zoology, University of Alberta, Edmonton, Alberta, Canada	Structure and ultrastructure of lymphoid anlagen
Perry Scheinok	Hahnemann Medical College, Hospital of Philadelphia, Philadelphia, Pennsylvania	Mathematical models in medical diagnosis
Giorgio Schreiber	Universidade Federalde Minas Gerais, Minas Gerais, Brazil	Slides of Belo Horizonte
Verne Schumaker	University of California, Los Angeles	Phase transition during centrifugation of high molecular weight DNA
Fabio Sereni	Universita di Milano, Milano, Italy	Nuclear RNA synthesis in the liver of the rat in the perinatal period: the role of corticosteroids
Russell W. Shaedler	Rockefeller University, New York	Bacterizing the germ-free gut
Frank W. Stahl	Institute of Molecular Biology, University of Oregon, Eugene	Gene clusters in bacteriophage T4
Pat Walne	The University of Tennessee, Knoxville	The effects of colchicine on cellular organization in *Chlamydomonas eugametos*
Hubert Wartenberg	Department of Biological Structure, University of Washington School of Medicine, Seattle	Some studies on the fine structure of two circumventriculary organs of the mammalian brain, epiphysis cerebri, and area postrema
Bruce L. Welch	The University of Tennessee Memorial Research Center and Hospital, Knoxville	Biochemical and morphological effects of social stimulus and their implications for population feedback control
John S. Wellington	University of California San Francisco Medical School, San Francisco	The relationship of late radiation changes in bone marrow to radiation leukemia

SCHEDULED SPEAKERS FOR PROFESSIONAL MEETINGS, SPRING–SUMMER 1967

CIBA Symposium on Cell Differentiation, London, England – Tuneo Yamada

11th Annual Meeting of the Biophysical Society, Houston, Texas – G. B. Cline (coauthors, Manuel Rosenbaum and N. G. Anderson), J. S. Cook, J. E. Donnellan (R. S. Stafford and J. E. Donnellan), S. P. Leibo (coauthor, Peter Mazur), R. A. McGrath (coauthor, R. W. Williams), R. O. Rahn (coauthor, J. L. Hosszu), Jane K. Setlow (coauthor, F. J. Bollum), R. B. Setlow (coauthors, W. L. Carrier and R. W. Williams), and P. A. Swenson

4th Annual Electron Microscopy Symposium of the Louisiana Society for Electron Microscopy, New Orleans, Louisiana – W. W. Harris (coauthors, Frances L. Ball, Helen Coates, G. B. Cline, N. G. Anderson, and R. M. Chanock)

International Symposium on Germfree Life Research, Nagoya, Japan – M. L. Simmons (coauthors, C. B. Richter and R. W. Tennant)

4th Basel Colloquium on Mammalian Sex Chromosomes in Differentiation and Development, Basel, Switzerland – Liane B. Russell

20th Annual Biology Research Conference, Gatlinburg, Tennessee – Rhoda F. Grell

58th Annual Meeting of the American Association for Cancer Research, Chicago, Illinois – Stanfield Rogers (coauthor, J. H. Evans) and A. C. Upton (coauthor, J. W. Conklin)

51st Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois – W. E. Barnett (coauthors, J. L. Epler and D. H. Brown), C. C Congdon (T. J. Mitchell, D. A. Gardiner, M. A. Kastenbaum, and C. C Congdon), F. J. Finamore (J. S. Clegg, A. H. Warner, and F. J. Finamore), Joan Wright Goodman (coauthors, Gayle C. Bosma and Sarah G. Shinpock), K. Bruce Jacobson (coauthor, J. B. Murphy), F. T. Kenney, T. T. Odell, Jr. (coauthor, C. W. Jackson), John Papaconstantinou (J. A. Stewart and John Papaconstantinou), D. C. Vann, L. C. Waters (coauthors, Elliot Volkin and G. David Novelli), and J. F. Weiss (coauthors, A. D. Kelmers and M. P. Stulberg)

Bone Marrow Conference Group, Chicago, Illinois – C. C Congdon

The Association of Southeastern Biologists, Columbia, South Carolina – P. W. Whiting

International Conference on Cell Synchrony, Oak Ridge, Tennessee – H. I. Adler (coauthors, W. D. Fisher and Amikam Cohen), F. J. Finamore, Alexander Hollaender, R. F. Kimball, G. J. Stine, and G. L. Whitson

67th Annual Meeting of the American Society for Microbiology, New York, New York – H. I. Adler (coauthors, Amikam Cohen, W. D. Fisher, and Alice A. Hardigree), D. E. Axelrod (coauthor, H. I. Adler), Amikam Cohen (coauthors, H. I. Adler, W. D. Fisher, and F. W. Shull), Roy Curtiss III (coauthors, J. A. Mays and D. R. Stallions), A. P. Harrison, Jr. (coauthor, Vivian E. Raabe), Alfred Hellman (coauthors, L. J. Wopschall and J. G. Farrelly), Kiki B. Hellman (coauthors, Alfred Hellman, R. B. Cumming, and G. David Novelli), L. R. Shugart (coauthor, M. P. Stulberg), R. L. Tyndall (coauthors, J. A. Otten, Ernestine Teeter, and N. D. Bowles), and C. J. Wust (coauthor, D. G. Ridner)

15th Annual Meeting of the Radiation Research Society, San Juan, Puerto Rico – M. A Bender (coauthors, F. J. de Serres, P. Carolyn Gooch, Ida R. Miller, and Sohei Kondo), N. K. Clapp (coauthors, A. W. Craig and A. C. Upton), G. E. Cosgrove (coauthors, A. C. Upton and C. C Congdon), E. B. Darden, Jr. (coauthors, K. S. Christenberry, M. C. Jernigan, J. W. Conklin, R. S. Bender, and A. C. Upton), H. V. Malling (coauthor, F. J. de Serres), J. L. Montour, M. L. Randolph, J. D. Regan (coauthors, J. E. Trosko), L. H. Smith (coauthor, T. W. McKinley, Jr.), A. C. Upton (coauthor, J. W. Conklin), R. C. von Borstel (coauthors, R. H. Smith and Anna R. Whiting), H. E. Walburg, Jr. (coauthors, G. E. Cosgrove and A. C. Upton), and Niel Wald (coauthors, S. Pan, A. C. Upton, and R. C. Brown)

1st International Congress of the Transplantation Society, Paris, France – C. C Congdon, Joan Wright Goodman

12th Annual Meeting of the Health Physics Society, Washington, D.C. – W. L. Russell and A. C. Upton

IAEA Study Group Meeting on the Effects of Ionizing Radiations on Meiotic Systems, Vienna, Austria – E. F. Oakberg, Liane B. Russell

International Union of Biochemistry and Seventh International Congress of Biochemistry, Tokyo, Japan – M. I. Dolin, R. K. Fujimura (coauthor, Elliot Volkin), K. Bruce Jacobson (coauthor, Virginia L. Holten), F. T. Kenney, J. X. Khym (coauthor, Mayo Uziel), G. David Novelli (coauthors, A. D. Kelmers, J. F. Weiss, C. Hancher, H. Weeren, and E. F. Phares), and M. P. Stulberg (coauthor, J. W. Longworth)

Foreign Travel

More than 20 members of the Biology Division visited abroad during the period August 1966–January 1967. Their activities, which took them to 20 foreign countries, ranged from participation at conferences and meetings to giving invited talks at various research institutions, colleges, and universities. Countries visited were:

Europe – Austria
Czechoslovakia
England
France
Germany
Greece
Italy
The Netherlands
Sweden
Switzerland
Yugoslavia

Asia – India
Israel
Japan
Pakistan
Thailand

Australia – Commonwealth of Australia

The Americas – Canada
Mexico
Venezuela

Eight Division members – L. G. Caro, Dorothy S. Cook, J. S. Cook, J. E. Donnellan, Jr., J. W. Longworth, John Papaconstantinou, R. O. Rahn, and B. K. Zimmerman – traveled to Vienna, Austria, in early September to participate in the Second International Biophysics Congress. Each investigator presented a paper at the Congress; titles and coauthors will be found in the **Lectures** section of this report.

Representing the Division at the Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, November 28–December 1, were Alexander Hollaender, D. D. Holten, Virginia L. Holten, F. T. Kenney, G. David Novelli, M. P. Stulberg, Elliot Volkin, and Craig Whitmire, Jr. See **Meetings and Conferences,** 1966, p. 32 for details.

R. A. Brown attended the 1966 Leukocyte Culture Conference, October 13–16, in Montreal, Canada, as a representative of the Division's Radiation Immunology section.

E. H. Y. Chu, who returned in September from a year's leave at the Karolinska Institute in Stockholm, Sweden, again traveled to Stockholm on January 18, 1967, for a month's stay. He will be doing collaborative research on tumor immunology.

L. G. Caro, who has been on leave of absence to Hammersmith Hospital, London, since June 1966, was in Naples, Italy, September 24–26, for the European Phage Meeting, at which he presented a paper entitled "The attachment of the DNA bacteriophage f1 to male strains of *E. coli.*" From Naples he traveled to Geneva, Switzerland, to confer with Dr. Edouard Kellenberger of the Institute of Molecular Biology, University of Geneva, concerning a course in electron microscopy to be offered by the Biology Division in the summer of 1968. He returned to London on October 4.

After participating in the Second Biophysics Congress in Vienna, Dorothy S. Cook and J. S. Cook visited various research institutions in France before returning to Oak Ridge on September 26.

J. E. Donnellan, Jr., visited the Institute of Microbiology of the Czechoslovak Academy of Sciences and the Department of Genetics, Microbiology, and Biology at Charles University, both in Prague, Czechoslovakia, in September. Then he traveled to France and The Netherlands, giving an invited lecture at the Institut Pasteur in Paris and at the Medical-Biological Laboratory RVO-TNO in Rijswijk.

Joan Wright Goodman, who spent six months in the Weizmann Institute of Rehovoth, Israel, traveled from there to Sydney, Australia, before returning to the Division. In Australia she attended the Congress of the International Society of Hematology and the Congress of the International Society of Blood Transfusion, August 21–29. At the latter she gave a paper on "Modification of poor growth of parental hemopoietic cells."

Alexander Hollaender, before attending the Symposium on Enzymatic Aspects of Metabolic Regulation in Mexico City, traveled to Caracas, Venezuela, where he met with Drs. Marcel Roche and Carlos Rivas to prepare the program for an international symposium on Basic Mechanisms in Photobiology to be held in Caracas in December 1967. R. B. Setlow, of the Biology Division, also attended the program-planning meetings. While in Venezuela, Dr. Hollaender visited universities in the cities of Cumaná and Maracaibo. On January 1, 1967, he began a foreign tour which will include visits to Switzerland, Greece, Thailand, India, Pakistan, Israel, Italy, and France. Dr. Hollaender will make a comprehensive survey of current radiation biology research and give numerous invited lectures and seminars.

Before returning to the Division from his year's stay at the Institute of Genetics in Rome, Italy, D. L. Lindsley spent August 1–6 in Germany and The Netherlands, where he lectured at the University of Munich, Germany; the Max Planck Institute for Biology in Tubingen, Germany; and at the University of Leiden, Leiden, The Netherlands. See **Lectures** for titles.

During September, J. W. Longworth visited laboratories in Germany, Switzerland, and France after attending the Biophysics Congress in Vienna. While in France, he lectured on "Luminescence and E.S.R. of nucleic acid protein" at the Institute for Macromolecular Research, Centre National de la Recherche Scientifique, Strasbourg.

Peter Mazur traveled in Japan and Australia during the month of August, presenting a paper entitled "Physical-chemical basis of injury from intracellular freezing in yeast" at the International Conference on Cryobiology held in Sapporo, Japan, August 14–17, and one on "Physical and chemical changes during freezing and thawing of cells" at the Symposium on New Methods of Preservation of Blood and Bone Marrow, XI Congress of the International Society of Blood Transfusion, Sydney, Australia, August 24–29. He visited laboratories in both countries and gave invited lectures at the CSIRO Division of Food Preservation in Sydney and at the University of Melbourne, Melbourne, Australia.

E. F. Oakberg attended the Colloquium on Physiology and Reproduction in Mammals, Paris, France, November 16–19, and presented an invited paper entitled "RNA metabolism in mammalian oocytes."

T. T. Odell, Jr., was in Sydney, Australia, August 21–26, to present the paper "How do megakaryocytes differentiate?" at the Congress of the International Society of Hematology.

Before traveling to Vienna for the Biophysics Congress, John Papaconstantinou attended both the Symposium on the Biochemistry of the Eye at Tutzing, Germany, August 9–14, and the International Congress of Ophthalmology, Munich, Germany, August 14–19. At the former, he delivered a paper on "An analysis of the ribosomal breakdown in lens fiber cell differentiation."

E. H. Perkins visited laboratories in Belgrade and Zagreb, Yugoslavia, September 2–6, to discuss problems of mutual interest with various Yugoslavian investigators. He then traveled to Como, Italy, for the International Symposium on Atherosclerosis and the Reticuloendothelial System. Before returning to Oak Ridge, he visited the Schweizerisches Forschungsinstitut, Medizinische Abteilung, Davos-Platz, Switzerland, and the Institut Pasteur and College de France, Paris, France. During his trip, Dr. Perkins presented papers at the Institut Ruder Boškovic, in Zagreb, and at the Symposium in Como. Titles and coauthors will be found in the **Lectures** section of this report.

After participating in the Biophysics Congress in Vienna, R. O. Rahn spent two weeks in September visiting laboratories in Germany, Switzerland, and France. He spoke on "Excited state properties of

polynucleotides at 77°K" at the Institute for Macromolecular Research, Centre National de la Recherche Scientifique, Strasbourg.

R. B. Setlow spent November 21–22 in Caracas, Venezuela, where he joined Alexander Hollaender of the Biology Division and Drs. Marcel Roche and Carlos Rivas of Venezuela in preparing a program for the planned International Symposium on Basic Mechanisms in Photobiology to be held in Caracas in December 1967.

Tuneo Yamada presented a paper entitled "Lens cell differentiation" at the Conference on Morphological and Biochemical Aspects of Cytodifferentiation, Valbella, Switzerland, September 8–10, and served as chairman of the session on the Central Nervous System.

Meetings and Conferences

1966

MEETING ON THE USE OF DNA OR RNA TO SUPPLEMENT THE HOST GENOME, PERHAPS USING VIRUSES AS VECTORS

An informal discussion meeting on the **Use of DNA or RNA to Supplement the Host Genome, Perhaps Using Viruses as Vectors** was organized by Stanfield Rogers of the Biology Division and held at Rockefeller University, New York City, on October 1. Other Division members attending were Alexander Hollaender and J. L. Liverman.

FALL MEETING OF BIOMEDICAL PROGRAM DIRECTORS

Alexander Hollaender attended the fall meeting of the Biomedical Program Directors of the U. S. Atomic Energy Commission held in Oak Ridge, October 10–11. The Health Physics Division, ORNL, served as host. These meetings are held three times each year to review research in the various biomedical programs at Commission laboratories.

SYMPOSIUM ON ENZYMATIC ASPECTS OF METABOLIC REGULATION

Seven Biology Division investigators – Alexander Hollaender, Virginia L. Holten, D. D. Holten, F. T. Kenney, G. David Novelli, M. P. Stulberg, and Elliot Volkin – attended the conference on **Enzymatic Aspects of Metabolic Regulation** held in Mexico City from November 28 to December 1. This was the sixth in the series of Latin American conferences held annually since 1961 under the sponsorship of scientific organizations in the host country with the encouragement of the Biology Division and with cosponsorship by the Division and other scientific and educational institutions in the United States.

Drs. Hollaender, Kenney, and Novelli were members of the organizing committee for the conference. In addition, Dr. Hollaender served as chairman of the closing session and gave the closing remarks, F. T. Kenney and Elliot Volkin presented papers, and M. P. Stulberg and G. David Novelli were discussants. Dr. Stulberg is serving as scientific editor of the proceedings, which are to be published as a **Journal of the National Cancer Institute Monograph**. Craig Whitmire, Jr., Division editor, also attended the conference to assist in preparation of the proceedings for publication.

Twenty-six papers, seven discussion papers, an opening and a closing lecture, and closing remarks were presented at the six sessions. Speakers included scientists from Argentina, Brazil, Chile, England, and France, as well as Mexico and the United States. Registered and attending the conference were additional investigators from the countries listed for the speakers; from Costa Rica, El Salvador, Guatemala, and Panama in Central America; and from Colombia, Venezuela, Peru, and Uruguay in South America. There were also many students from Mexico at various sessions of the symposium.

Symposium sponsors were the National University of Mexico; The National Polytechnic Institute; the University of Wisconsin; Biology Division, ORNL; U.S. Atomic Energy Commission; The National Science Foundation; Ford Foundation; and the U.S. National Institutes of Health.

BONE MARROW CONFERENCE

A midwinter bone marrow conference was held December 2–3 at the University of Michigan in Ann Arbor under the chairmanship of Dr. Byron S. Berlin. Division members C. C Congdon and R. A. Popp were included among the speakers, and the latter also served as chairman of one of the sessions. Abstracts of the papers presented at the meeting, as well as several contributed papers, appear in *Experimental Hematology* No. 12.

This conference was the most recent in a series of bone marrow transplantation and chemical protection meetings, first held in 1957 in Oak Ridge, arranged by Biology Division investigators with the cooperation of many scientists and institutions throughout the world to provide a means for prompt exchange of the latest experimental information between clinical and animal researchers.

ANNUAL INFORMATION MEETING

The Oak Ridge National Laboratory Advisory Committee for Biology made its annual visit to the Division December 7–9. The committee members are 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.

During the first two days of the **Annual Information Meeting** Division investigators presented summaries of their work at formal sessions. Discussion areas covered included developmental biology, biochemistry, biophysics, molecular anatomy, genetics, carcinogenesis, immunology, pathology and physiology, mammalian recovery program and experimental animal facility, and educational activities. After the formal sessions, and on the last day, committee members visited individual laboratories and participated in informal discussions.

1967

TWENTIETH ANNUAL BIOLOGY RESEARCH CONFERENCE

The 1967 Research Conference, sponsored by the Biology Division, ORNL, will be held April 10–13 at the Riverside Motor Lodge in Gatlinburg, Tennessee. The organizing committee, R. F. Kimball (chairman), Roy Curtiss III (cochairman), C. M. Steinberg, Elliot Volkin, and R. C. von Borstel, has announced the following program:

CHROMOSOME MECHANICS AT THE MOLECULAR LEVEL

Monday, April 10

Opening Remarks – Alexander Hollaender

Welcome Address – H. G. MacPherson, Deputy Director
Oak Ridge National Laboratory

Introduction – R. F. Kimball

Morning Session

Chairman: Enrico G. Calef, Laboratorio Internazionale di Genetica e Biofisica, Naples, Italy

Circular Genetic Maps – Franklin W. Stahl, Institute of Molecular Biology, University of Oregon, Eugene

Systematics of Viral DNA Molecules – C. A. Thomas, Jr., The Thomas C. Jenkins Department of Biophysics, The Johns Hopkins University, Baltimore, Maryland

Afternoon Session

Chairman: Karl G. Lark, Department of Physics, Kansas State University, Manhattan

Replication of RNA – Sol Spiegelman, Department of Microbiology, University of Illinois, Urbana

Mechanics of DNA Replication in Bacteria – John Cairns and Cedric Davern, Cold Spring Harbor Laboratory of Quantitative Biology, Cold Spring Harbor, New York

Tuesday, April 11

Morning Session

Chairman: R. L. Sinsheimer, Division of Biology, California Institute of Technology, Pasadena, California

Regulation of Replication Studied in Bacterial Episomes: On the Mechanism of Genetic Transfer by F^+ or Hfr Strains of Escherichia coli – François Cuzin, Commissariat à l'Energie Atomique, Service de Biochimie, Gif-sur-Yvette, France, and François Jacob, Institut Pasteur, Paris

DNA Replication and Recombination in Eukaryotes – Kwen-Sheng Chiang and Noboru Sueoka, Department of Biology, Princeton University, Princeton, New Jersey

Afternoon

(Free)

Wednesday, April 12

Morning Session

Chairman: A. H. Sturtevant, Kerckhoff Biological Laboratories, California Institute of Technology, Pasadena

Pairing at the Molecular Level – Matthew S. Meselson, Biological Laboratories, Harvard University, Cambridge, Massachusetts

Pairing at the Chromosomal Level – Rhoda F. Grell, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Afternoon Session

Chairman: Julian Gross, Microbial Genetics Research Unit, Hammersmith Hospital, London

Genetic Recombination in Phage – A. H. Doermann and David H. Parma, Department of Biology, University of Washington, Seattle

A Genetic Analysis of Recombination Proficiency – A. J. Clark, Department of Molecular Biology, University of California, Berkeley

Reactions Occurring at Termini in DNA – Jerard Hurwitz, M. L. Gefter, and A. J. Becker, Department of Molecular Biology, University of Washington, Seattle

Reactions Occurring at Termini in DNA – Jerard Hurwitz, M. L. Gefter, and A. J. Becker, Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York

Thursday, April 13

Morning Session

Chairman: A. D. Hershey, Genetics Research Unit, Carnegie Institution of Washington, Cold Spring Harbor, New York

Molecular Mechanisms of Genetic Recombination – Jun-ichi Tomizawa, Department of Chemistry, National Institute of Health, Shinagawa-ku, Tokyo, Japan

Perspectives – R. D. Hotchkiss, Rockefeller University, New York

BIOMEDICAL PROGRAM DIRECTORS MEETINGS

J. L. Liverman and R. F. Kimball will attend the winter meeting of the Biomedical Program Directors of the United States Atomic Energy Commission on February 13–14 in New York City. The spring meeting will be April 24–25 at Livermore, California.

BONE MARROW CONFERENCES

A series of three bone marrow transplantation and chemical radiation protection conferences is being planned for 1967. The first will be held on April 17 in conjunction with the Federation of American Societies for Experimental Biology meeting in Chicago. Dr. Jerry P. Lewis of Presbyterian–St. Luke's Hospital, Chicago, is organizing the 2-hr evening discussion session.

Preparations for a 1-day bone marrow conference in Paris are being made by Drs. Georges Mathé and Leon Schwartzenberg of the Institut de Cancerologie et d'Immunogénétique. This meeting will take place on June 26 in connection with, and immediately preceding, the First International Congress of the Transplantation Society, June 27–30.

The final conference of the year will be held in Oak Ridge in midwinter and will review ten years of research in bone marrow transplantation and other investigations related to radiation protection and recovery.

2ND INTERNATIONAL CONFERENCE ON CELL SYNCHRONY

Over 100 scientific investigators from the United States and abroad will participate in the **2nd International Conference on Cell Synchrony** to be held in Oak Ridge on April 27–29. The organizing committee – G. L. Whitson (Biology Division, Oak Ridge National Laboratory) and G. M. Padilla (Wrightsville Biomedical Marine Laboratory, Wilmington, North Carolina), cochairmen; W. D. Fisher (Biology Division), and I. L. Cameron (Upstate Medical Center, State University of New York, Syracuse) – has arranged a program consisting of 39 invited papers and 9 discussion periods. Session topics are "Genetic studies in cell synchrony," "Developmental aspects of cell synchrony," "Regulation and control in synchronized cells," "Biochemistry and physiology of synchronized cells," and "Macromolecular aspects of cell division synchrony."

R. F. Kimball, Director, Biology Division, will present the opening remarks and Alvin M. Weinberg, Director of the Oak Ridge National Laboratory, will deliver the welcoming address. Alexander Hollaender, Biology Division, will serve as conference chairman and present introductory remarks, and J. M. Mitchison of the University of Edinburgh, Edinburgh, Scotland, will give the summation. Division members scheduled to present papers or serve as invited discussants are H. I. Adler (coauthors, W. D. Fisher and Amikam Cohen), F. J. Finamore, G. J. Stine, and G. L. Whitson.

THIRD CONFERENCE ON BLOOD PLATELETS

Plans are being made by T. T. Odell, Jr., of the Biology Division, and Robert M. Heyssel, M.D., Vanderbilt University School of Medicine, to hold the **Third Conference on Blood Platelets** in Oak Ridge on June 22–23. Investigators working on the functions and characteristics of blood platelets will present papers and discuss recent developments in this area of research. Abstracts of the papers will be published in *Blood.*

EIGHTH ANNUAL SYMPOSIUM OF THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

Cytodifferentiation with Special Reference to Immunology will be the subject of the **Eighth Annual Symposium of the International Society for Cell Biology** to be held October 9–13 in Gatlinburg, Tennessee. Takashi Makinodan and John Papaconstantinou are assisting in planning the meeting and are making arrangements for participants to visit with Biology Division staff members in their laboratories in Oak Ridge. There will be nine half-day sessions during which 20 to 25 papers will be presented on the general topics "Use of immunologic techniques (indicator assay)" and "Immunologic response as a model system to study differentiation."

INTERNATIONAL SYMPOSIUM ON BASIC MECHANISMS IN PHOTOBIOLOGY

The seventh in the series of Latin American conferences organized by Alexander Hollaender and cosponsored by the Biology Division will be held in Caracas, Venezuela, in December 1967. Drs. Hollaender and R. B. Setlow were in Caracas, November 21–22, 1966, to meet with Drs. Marcel Roche and Carlos Rivas and make final plans for the symposium, **Basic Mechanisms in Photobiology**. A tentative program is being prepared and will be announced in the next semiannual progress report (July 31, 1967).

Educational Activities

GRADUATE COURSES TAUGHT

UT-ORNL Ford Foundation Program – H. I. Adler has continued to participate in the University of Tennessee–Oak Ridge National Laboratory Cooperative Research Program sponsored by the Ford Foundation, teaching a course in Bacterial Genetics for the University's Department of Microbiology during the fall and winter quarters. This course has as its primary concerns the physical basis of genetics, bacterial cytology, mutations, DNA as genetic material, and genetic systems of bacteria and bacterial viruses.

C. C Congdon taught a course dealing with Pathological Processes at the University of Tennessee during the winter quarter. The course will be continued in the spring.

Also under the UT-ORNL Ford Foundation Program, A. H. Haber will teach a course entitled "Developmental Plant Physiology" during the spring quarter.

Also under this program, L. H. Smith – together with R. W. Holton, Head of the University's Department of Botany – is teaching a one-quarter course in Cell Physiology which concentrates on the functional activities of cells, with special emphasis on the physiochemical phenomena of protoplasm; on organization in the cell; and on the transport of materials and the release, sources, and uses of energy in the cell.

UT–Oak Ridge Resident Graduate Program – The following courses have been taught by Division members through the UT–Oak Ridge Resident Graduate Program:*

Advanced Genetics. – A course in Advanced Genetics is being taught this winter by W. E. Barnett. The course places major emphasis on modern trends and theories in genetics.

Cellular and Comparative Biochemistry. – F. J. Finamore taught a course in Cellular and Comparative Biochemistry during the fall quarter, and is currently teaching the same course. Topics covered include intermediary metabolism energy transformations, the properties of enzymes, and enzyme kinetics.

Concepts in General Zoology. – Organized in 1964 by G. L. Whitson and N. G. Anderson, the General Zoology course was taught during the fall quarter by Dr. Whitson and is being taught by him again this winter. Designed specifically to train Oak Ridge nonbiological personnel in basic biology, this course is conducted on a level appropriate to college graduates with no prior training in zoology, with emphasis being placed on such problems as animal complexity, reproduction, cell differentiation, subcellular organization, and genetics.

General Genetics. – W. E. Barnett taught a course in General Genetics during the fall quarter. This course dealt mainly with the fundamental principles of heredity.

Human Genetics. – A course in Human Genetics is currently being taught by J. D. Regan; it 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.

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*All UT–Oak Ridge Resident Graduate Program courses are taught at the ORAU Special Training Building, Laboratory Road.

LECTURES PRESENTED IN COURSES

On August 16, U. H. Ehling and A. C. Upton lectured to a group of teachers from various state universities and junior colleges attending an AEC-NSF Basic Institute for Nuclear Science and Engineering. The six-week program, held at North Carolina State University, included a week's visit to Oak Ridge during which Dr. Ehling spoke on "Radio-genetics" and Dr. Upton spoke on "Radio-somatics."

During the period September 19–January 31, the following Division members participated as guest lecturers in G. L. Whitson's General Zoology course: N. G. Anderson, W. E. Barnett, D. G. Doherty, W. D. Fisher, W. D. Gude, and R. A. Popp.

J. F. Albright and N. G. Anderson lectured on November 30 and December 9, respectively, to reserve officers from Army, Navy, and Air Force research and development organizations attending the 16th Nuclear Science Seminar, ORAU. Albright's topic was "Immunology and scientific research," and Anderson spoke on "The Molecular Anatomy Program."

W. E. Cohn was at Rockefeller University, New York, December 12–16, as a Visiting Professor. He gave two lectures in a course on Nucleic Acids, and presented a seminar entitled "Some recent developments in chromatography."

W. D. Gude lectured on "Autoradiography" to a group taking the ORAU Medical Radioisotopes Course on October 17. In addition to the lecture, he conducted the group on a tour of the Biology Division.

K. B. Jacobson served from December 12 to 14 as guest lecturer in a Marquette University graduate course; his topic was "tRNA structure and function."

On November 29, R. A. Popp gave a seminar at the University of Michigan on "Esterase variants in mice" as part of a joint Botany-Zoology graduate course.

On September 30, W. L. Russell spoke on "Radiation biology – genetic effects" to participants in the ten-week Health Physics Course, ORAU. On October 3, G. E. Cosgrove lectured to the same group on "Delayed somatic effects of radiation."

COURSES TAKEN

M. L. Simmons attended the Medical Genetics Course given at Bar Harbor, Maine, from July 31 through August 13.

J. L. Liverman attended a course in Comparative Pathobiology sponsored by the Committee on Pathology of the National Research Council, Aspen Biological Institute, and the University of Colorado, and given at Aspen, Colorado, from August 7 through August 17.

C. B. Richter was in Washington, D.C., September 18–23, to attend the course on Pathology of Laboratory Animals at the Armed Forces Institute of Pathology.

Division Members Working in Other Laboratories

LEAVE OF ABSENCE

J. G. Brewen is in Canberra, Australia, where he is working with Drs. W. J. Peacock and Richard Brock of the Commonwealth Scientific Research Organization on radiation-induced chromosome aberration studies. Dr. Brewen's one-year stay in Canberra is partially supported by a U.S. Public Health Service Special Fellowship. He will return to the Division in September 1967.

L. G. Caro, who has been working on mechanisms of DNA transfer in bacterial conjugation and transduction at the Microbial Genetics Research Unit, Medical Research Council, Hammersmith Hospital, London, England, under an eight-month Senior Postdoctoral Fellowship granted by the National Science Foundation, will return to the Laboratory early in March.

J. G. Farrelly was awarded a National Science Foundation Predoctoral Fellowship to carry on graduate study in biochemistry at the University of Tennessee. He is on leave from the Inhalation Carcinogenesis group while continuing his studies.

V. K. Jenkins is continuing his studies and research in zoology at the University of Tennessee. He is on leave from the Pathology and Physiology group under a U.S. Public Health Service Predoctoral Fellowship.

G. E. Stapleton, a member of the Radiation Biology group, is at the Division of Biology and Medicine of the U.S. Atomic Energy Commission in Washington, D.C., on a two-year leave of absence.

COLLABORATIVE RESEARCH

E. H. Y. Chu is spending the period January 18–February 24 at the Karolinska Institute, Stockholm, Sweden, to extend studies in mammalian cytology and human cytogenetics initiated during his previous stay at the Swedish institution (September 1965–August 1966). This research is being conducted in collaboration with Dr. George Klein of the Karolinska Institute.

Cytology and Genetics

R. F. Kimball[a]

Effects of Radiation on Paramecium (1.1–1.3)

R. F. Kimball[a]
G. L. Whitson
E. G. Bailiff[a]
Barbara R. Bussell[a]
A. A. Francis
Stella W. Perdue
Carrie L. Wells[a]
Betsy M. Sutherland[b]
Sandra F. Bowles[c]
Kathleen L. Grandi[d]
D. H. Lauer[d]

Molecular Photobiology of DNA (1.4–1.7)

Jane K. Setlow
Amir Muhammed[e]
M. E. Boling
Dianne C. Brown

Mammalian Genetic Cytology

E. H. Y. Chu[a]
E. Virginia Merriam[f]
Tatsuo Omura[e]
J. E. Trosko
Patricia A. Brimer
Lana T. Covington[a]
Nina S. Hammer[a]

Mammalian Biochemical Genetics (1.8, 1.9)

R. A. Popp
W. S. McEwen
Diana M. Popp

Rhynchosciara Genetics (1.10–1.12)

M. Ellen Mattingly
Crodowaldo Pavan[g]
Günter Weirich[e]
Shirley P. Ogle
C. L. Parker
A. L. P. Perondini[e]
L. C. G. Simoes[e]
Vivien B. Whitfield[d]

Participation in Cooperative Programs:

AEC-NIH Chemical Carcinogenesis (22.10, 22.11)

[a]Dual assignments
[b]ORAU Predoctoral Fellow
[c]Loanee
[d]Student trainee
[e]Visiting investigator from abroad
[f]USPHS Postdoctoral Fellow
[g]Consultant

1.1 PHOTOREACTIVATION OF PYRIMIDINE DIMERS IN *PARAMECIUM AURELIA* DNA

Betsy M. Sutherland[1] R. B. Setlow[2]

We showed previously[3] that mass cultures of paramecia can be heavily labeled by feeding on *E. coli* 15 T^- previously labeled with ^{3}H-thymidine. The paramecia are then grown with *Aerobacter aerogenes* for $2\frac{1}{2}$ hr to removed labeled DNA from the food vacuoles. Since there are three DNA's in the system, it is necessary to show that the majority of the labeled DNA examined for dimers is paramecium DNA. This has been done in two ways. (1) The tritiated DNA was extracted, exposed to 5000 ergs/mm^2 of 2800-A ultraviolet radiation, hydrolyzed, chromatographed, eluted, and counted. The observed ratio of uracil-thymine to thymine-thymine dimers corresponded to a DNA containing about 67% A-T. The A-T content of *P. aurelia* is 70%, while that of *E. coli* is 50% and that of *A. aero-*

genes 45%. (2) The DNA extracted from the cultures of Paramecium was centrifuged in an angle head rotor to equilibrium in CsCl together with a ^{14}C-labeled *E. coli* DNA marker. Assays of fractions from the gradient showed the presence of three DNA's of densities 1.69 (Paramecium), 1.71 (*E. coli*), and 1.72 (Aerobacter). About 95% of the tritium label was associated with DNA from *P. aurelia*.

We have also investigated the disappearance of uv-induced pyrimidine dimers upon exposure to photoreactivating light. The paramecia, previously labeled for several generations with tritiated thymidine, were exposed to 3,000 ergs/mm^2 of 2537-A ultraviolet radiation, the highest exposure found to yield 100% survival at 48 hr. They were then given exposures of photoreactivating light ranging from 0 to 400,000 ergs/mm^2. Dimer content in the trichloroacetic (TCA) insoluble fraction decreased with increasing exposure to photoreactivating light; cells given no photoreactivating light showed only a slight loss of dimers. There was no change in the dimer content of the TCA-soluble fraction. Thus it appears that in the presence of photoreactivating light, pyrimidine dimers are monomerized in Paramecium.

References

[1]Predoctoral fellow, Oak Ridge Associated Universities.

[2]Biophysics section.

[3]Betsy M. Sutherland and W. L. Carrier, *Biol. Div. Semiann. Progr. Rept. July 31, 1966,* ORNL-3999, pp. 40–41.

1.2 THE REPAIR OF UV DAMAGE IN *TETRAHYMENA PYRIFORMIS* GL-C

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

Introduction. – We are continuing our investigations on the effects of ultraviolet light on cell division in *Tetrahymena pyriformis,* strain GL-C. In an earlier report we were able to identify thymine dimers chromatographically in the DNA hydrolysates of these cells.[2] Recently, we have been able to show that exposure of *Tetrahymena* to ultraviolet light, followed by growth in the dark, yields fewer thymine dimers in the DNA. In two separate experiments, using the same dose of ultraviolet light followed by exposure to photoreactivating light, no reduction in the amount of thymine dimers was evident. These results have prompted further investigation into this problem.

Methods. – Log phase cells 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 medium. Different samples of these cells were then given (1) a dose of 2000 ergs/mm^2 of 2652-A ultraviolet light, (2) 2000 ergs/mm^2 of 2652-A uv followed by growth in the dark in normal medium for 12 to 24 hr, and (3) 2000 ergs/mm^2 of uv followed by a dose of 4×10^5 ergs/mm^2 of photoreactivating light (3650 A). DNA hydrolysates were chromatographed two-dimensionally by butanol–acetic acid–H_2O and then butanol-H_2O.

Results and Discussion. – Thymine dimers have been identified chromatographically from DNA lysates of *Tetrahymena pyriformis* which were irradiated with 2000 ergs/mm^2 of uv at 2652 A. No thymine dimers have been observed in control samples. Cells irradiated with 2000 ergs/mm^2 of uv followed by 12 to 24 hr in the dark yielded fewer thymine dimers than the uv-irradiated sample. On the other hand, cells irradiated with 2000 ergs/mm^2 of uv and then followed by a dose of 4×10^5 ergs/mm^2 of visible light failed to show a reduction in thymine dimers as compared with the irradiated sample. It has been reported elsewhere,[3] however, that a dark repair mechanism is present in *Tetrahymena* (strain unreported), and it was further suggested that a reduction in the amount of this repair was due to photoreactivation involving cleavage of pyrimidine dimers. We have, however, obtained results from single studies which indicate that while photoreactivating light increases the delays in cell division it also increases the survival of previously uv-irradiated cells. These results clearly indicate that further experiments are necessary in order to elucidate the mechanism of repair of uv damage and photoreactivation in this organism.

References

[1]Biophysics section.

[2]G. L. Whitson, A. A. Francis, W. L. Carrier, and K. L. Grandi, *Biol. Div. Semiann. Progr. Rept. July 31, 1966,* ORNL-3999, p. 41.

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

1.3 CYCLIC CHANGES OF THE ALCOHOL-SOLUBLE CARBOHYDRATES IN SYNCHRONIZED *TETRAHYMENA*

G. L. Whitson J. G. Green[1]
A. A. Francis D. D. Willis[1]

It has been reported that the total alcohol-soluble sugar content in *Tetrahymena* decreases during the process of synchronization and that it remains low and fails to rise after cell division.[2] On the contrary, we have found that 15 alcohol-soluble carbohydrate compounds appear in cell extracts of *Tetrahymena* after synchronization.[3] The concentrations of most of these compounds increase to a peak prior to cell division, decrease during cell division, and are lower after cell division occurs. Two of these soluble components are maltose and glucose, as indicated by cochromatography with 18 known standards. The sequential changes of these alcohol-soluble compounds indicate that a complex interrelationship exists between the synthesis and utilization of soluble carbohydrates in synchronized cells. The possible role of some of these compounds in relation to cell division is being investigated. These studies have been made possible by the use of a prototype carbohydrate analyzer developed by J. G. Green.[4]

References

[1]AEC-NIH Molecular Anatomy Program.
[2]T. Kamiya, *J. Biochem.* **46**, 1187 (1959).
[3]O. H. Scherbaum and E. Zeuthen's method of synchronization, *Exptl. Cell Res.* **6**, 221 (1954).
[4]J. G. Green, *Natl. Cancer Inst. Monograph* **21**, 447 (1966).

1.4 THE MINIMUM SIZE OF THE SUBSTRATE FOR THE PHOTOREACTIVATING ENZYME

Jane K. Setlow F. J. Bollum[1]

Introduction. – Ultraviolet-inactivated transforming DNA is reactivated by illumination in the presence of photoreactivating enzyme from yeast. In this reaction uv-induced dimers between adjacent pyrimidines are split. The ability of a uv-irradiated synthetic compound to act as a substrate for the photoreactivating enzyme may be measured by observing the competition between the compound and transforming DNA for the enzyme. Determination of the competing ability of synthetic compounds of known length is a way of assaying the minimum size of the polynucleotide containing dimer which is a substrate for the enzyme.

Methods and Results. – Oligodeoxythymidylates were obtained by deoxyribonuclease I degradation of polythymidylate, which in turn was prepared with the terminal deoxynucleotidyl transferase from calf thymus gland. The deoxyribonuclease digest was separated into pieces of different lengths by means of a DEAE column, and length assignments were made on the basis of calling the first peak having initiator activity for the terminal transferase a trinucleotide. Other assignments were by assumption of a continuous series and from chromatographic identification.

When the chain contained 18 residues, the compound competed as well as a polydeoxythymidylate several hundred residues long. The oligomer with 14 residues competed less well, the 9-residue compound only slightly, and the pentamer not at all.

Conclusion. – An oligonucleotide chain must contain around nine nucleotides in order for binding to the photoreactivating enzyme to occur.

References

[1]University of Kentucky Medical Center, Lexington, Kentucky.

1.5 THE RELATIONSHIP BETWEEN CYTOCHROME b_2 AND PHOTOREACTIVATING ENZYME

Jane K. Setlow Dianne C. Brown

Introduction. – It was recently claimed by Duine and Berends[1] that the enzyme cytochrome b_2 from yeast is a photoreactivating enzyme, the evidence being that cytochrome b_2 preparations contained photoreactivating activity. We have investigated this claim in two ways: (1) we have measured the relative activities of photoreactivating enzyme and L(+)-lactate dehydrogenase (cytochrome b_2) in a number of yeast preparations, including highly purified cytochrome b_2 and highly purified photoreactivating enzyme, and (2) we have investigated by column chromatography the behavior of the two activities in a commercial preparation of cytochrome b_2 from the same firm that supplied Duine and Berends.

Methods and Results. – Photoreactivating enzyme was measured by its ability to photoreactivate uv-inactivated transforming DNA. Lactate dehydrogenase was assayed by observing spectrophotometrically the rate of reduction of ferricyanide in the presence of lactate. The two activities in the various yeast preparations were not correlated. The purest preparations of each enzyme contained no observable activity of the other enzyme. The commercial preparation exhibited on a phosphocellulose column three different peaks of lactate dehydrogenase activity. The photoreactivating activity could be separated from these. In addition, the two highly purified enzymes mixed together eluted at different alkali concentrations from a phosphocellulose column.

Conclusion. – Cytochrome b_2 is not photoreactivating enzyme.

References

[1]J. A. Duine and W. Berends, *Biochim. Biophys. Res. Commun.* **24**, 888 (1966).

1.6 ALCOHOL DEHYDROGENASE AND INVERTASE IN STATIONARY AND LOGARITHMICALLY GROWING YEAST

M. E. Boling Jane K. Setlow

Introduction. – The level of photoreactivating enzyme was found to be considerably higher in stationary cells than in logarithmically growing cells.[1] As a kind of control to this experiment, levels of two other enzymes were investigated as a function of growth state.

Methods and Results. – Extracts were made from six different yeast strains, including diploid and haploid *Saccharomyces cerevisiae* and *S. carlsbergensis*. Alcohol dehydrogenase (ADH) was measured by observing spectrophotometrically the rate of reduction of diphosphopyridine nucleotide in the presence of alcohol. Invertase was measured by assaying the rate of appearance of hydrolysis products of sugar with dinitrosalicylic reagent.

The specific activities of ADH were similar in extracts from all the strains (including a related diploid and haploid) and did not differ with growth stage. However, invertase behaved in these extracts like the photoreactivating enzyme in that the stationary cells contained a higher level of the enzyme.

References

[1]M. E. Boling and J. K. Setlow, *Biol. Div. Semi-ann. Progr. Rept. July 31, 1966*, ORNL-3999, pp. 43–44.

1.7 LOCALIZATION OF PHOTOREACTIVATING ENZYME IN A BACTERIAL CELL

Amikam Cohen[1] Jane K. Setlow H. I. Adler[1]

Introduction. – It is of interest to determine where enzymes are located in cells because of the general problem of cell organization and because such information may help in understanding how enzymes work. There is considerable evidence that the photoreactivating enzyme's sole substrate is uv-induced pyrimidine dimers in the cell's DNA. Therefore the question arises whether or not the enzyme is localized in the region of the cell containing the DNA. The answer to this question for one particular situation has been provided by experiments with *Escherichia coli* minicells (see paper 7.4) and their parent cells, the minicells being without DNA.

Methods and Results. – Minicells were separated from parent cells as described in paper 7.4 of this report. Separated cells and minicells were sonicated, and cell wall debris and unbroken cells were removed by centrifugation. Photoreactivating enzyme was assayed in the resulting extracts by its ability to increase transforming ability of uv-irradiated *Hemophilus influenzae* DNA under photoreactivating illumination. Photoreactivating enzyme was readily observable in the extracts from parent cells, but none could be detected in the minicell extracts.

Conclusion. – Photoreactivating enzyme in *Escherichia coli* is localized with the DNA.

References

[1]Radiation Microbiology and Microbial Genetics section.

1.8 TRANSPLANTATION ANTIGENS OF MOUSE SPLEEN CELLS[1]

R. A. Popp Diana M. Popp
N. G. Anderson[2] W. S. McEwen

Introduction. – Through the use of the zonal centrifuge, the subcellular particles that contain the

principal transplantation antigens (H-2 antigens) of the mouse have been isolated.[3] The region of the zonal cuts which contained the transplantation antigens was evaluated by hemagglutination inhibition of H-2 isoantibody.[3] Studies reported here show that the subcellular particles are capable of inducing isoantibodies when injected into allogeneic mice and that the antigen associated with the particles can be solubilized in Tris buffer.

Methods and Results. – Fractions of particulate material (100,000 × *g* pellet) recovered from the zonal centrifuge were pooled into six groups based on the profile of the absorption at 260 mμ of the effluent from the zonal centrifuge. Group I contained the pellet from cuts 1 through 7; group II, 8 to 11; group III, 12 to 17; group IV, 10 to 34; group V, 35 to 39; and group VI contained material removed from the wall of the rotor. Pooled material from the spleens of ten C57BL/6 mice was injected intraperitoneally into ten B10.D2 mice. Thus, each mouse received one spleen equivalent at each injection. The first injection was given on day 0. On day 7 each mouse received a second injection. No antibody was observed on days 11 and 13. A titer of 1:16 was noted in recipients of groups I and II at day 19. A third injection was given on day 24, and a titer of 1:128 appeared in recipients of groups I and II at day 34. No antibody was detected in mice receiving particles from groups III through VI.

Particles from group II, which contained most of the antigen detectable by hemagglutination inhibition,[3] were suspended in 0.05 *M* Tris buffer, pH 7.4, and allowed to stand at room temperature or 37° for several hours. The particles were removed by centrifugation, and the supernatant was dialyzed overnight and lyophilized. Hemagglutination inhibition assays indicated that about half of the antigen was removed from the particle and was present in the lyophilized supernatant. No difference was observed between aliquots allowed to stand at room temperature and 37°.

Discussion. – The antibody induction assay confirms the results of the hemagglutination inhibition studies and suggests that the antigen is associated with small particles that sediment in sucrose gradients less rapidly than microsomes, mitochondria, or large cell wall fragments, which appear in groups III–V. The detachment of the antigen from the particles is significant because the antigen must be solubilized in order to proceed with the purification of the antigen. Purification is an essential step prior to further studies on the physical and chemical properties of the transplantation antigens in the mouse.

References

[1]Research supported in part by the AEC-NIH Molecular Anatomy Program.

[2]AEC-NIH Molecular Anatomy Program.

[3]R. A. Popp, N. G. Anderson, and D. M. Popp, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1966,* ORNL-3922, p. 43.

1.9 POSSIBLE IDENTITY OF RHO AND "R" ANTIGENS IN MICE

Diana M. Popp G. D. Snell[1]

Introduction. – A non-H-2 red cell agglutinin has been described in strain RFM/Un and F/St mice and has been designated rho.[2] The antibody against rho has been demonstrated in C3H anti RFM and A.CA anti RFM antisera. Subsequently, Snell (personal communication) noted a similarity in strain distribution between rho and "R" and an antithetical relationship between rho or "R" and "Z" originally described by Hoecker.[3] This study was done to determine more directly whether rho and "R" might be identical and if "Z" is an allelic product.

Materials and Methods. – Antiserum A.CA anti RFM was used to define rho positive and rho negative animals. LP.RIII anti RIII was used to define "R" positive and "R" negative animals. A back cross population of (DBA/2 × RFM)F_1 × DBA/2 mice, which had been classified earlier for rho, were tested for their "R" phenotypes by the red cell agglutination technique of Gorer and Mikulska.[4] Also 23 strains of laboratory mice were tested for the presence of rho, "R," and "Z" antigens. RIII anti LP.RIII antiserum was used to detect "Z" antigen.

Results and Discussion. – Twenty-three backcross mice were classified with the anti "R" antiserum. Nine of these had been classified previously as being rho positive and 14 had been classified as rho negative. When tested with the anti "R" antiserum, all nine rho positive animals were also positive for "R" and all 14 of the rho negative animals were also negative for "R." Thus, the data indicate that rho and "R" are identical or products of

closely linked genes. The strain distribution showed that rho and "R" are present in strains RIII, RFM/Un, RF/J, and F/St, and that all other strains tested have "Z," which indicates that "Z" is quite likely an allelic product.

References

[1]The Jackson Laboratory, Bar Harbor, Maine.
[2]D. M. Popp, *Transplantation*, in press.
[3]G. Hoecker, O. Pizarro, and A. Ramos, *Transplantation Bull.* **6**, 407 (1959).
[4]P. A. Gorer and Z. B. Mikulska, *Cancer Res.* **14**, 651 (1954).

1.10 PATTERNS OF PUFF DEVELOPMENT IN RHYNCHOSCIARA

M. Ellen Mattingly C. L. Parker

The larval and pupal forms of the sciarid fly Rhynchosciara have several desirable characteristics not found elsewhere in insect populations. The comparatively enormous size of the larvae and their polytene chromosomes facilitate the study of chromosomal phenomena. Furthermore, during a period of approximately 40 days, the progeny of a single female develop synchronously, pupate in a common puparium, and emerge together from the puparium as adults of the same sex.

At the beginning of the pupation period the larvae form a tentlike net which progressively becomes more compact until each pupa is inclosed in a separate compartment of the puparium. During the elaboration of this puparium, the salivary gland cells are particularly active, and it is at this time that the polytene chromosomes develop their most elaborate puffs.

We have investigated the sequential appearance of the major puffs in each of the four chromosomes (A, B, C, and X). Maps for the banding pattern of each of the four chromosomes were first developed. The location of the puffs could then be related to the above map units. Fig. 1.10.1 represents schematically the sequence of events in one of the four chromosomes, that is, chromosome A. This is the only chromosome of Rhynchosciara for which a map had previously been published; the terminology from that published map has been retained.[1]

ORNL-DWG 67-2821

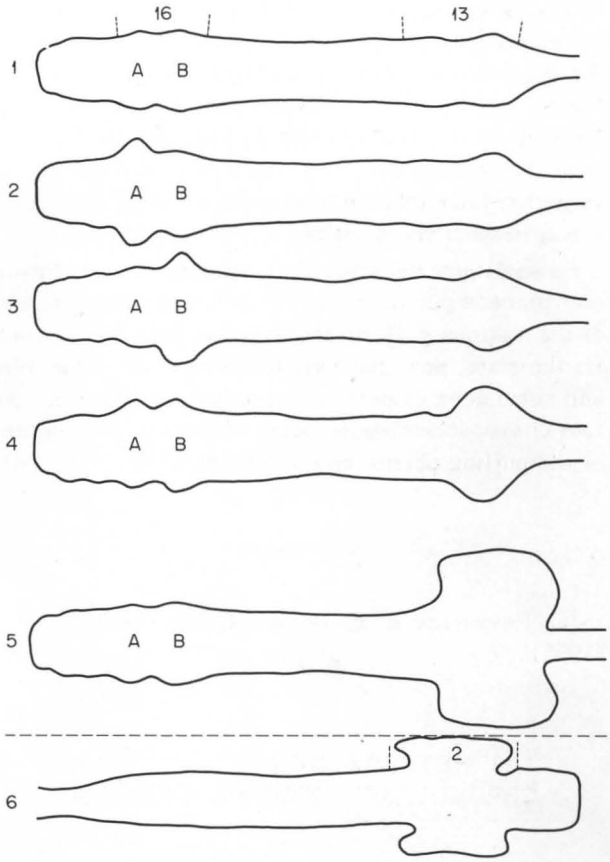

Fig. 1.10.1. A Schematic Representation of the Appearance of the Major Puffs on Chromosome A of Rhynchosciara. 1–5, left arm of chromosome A; 6, right arm of chromosome A.

Three major puffs develop on the left arm of chromosome A. The first two appear in segment 16 as the larvae begin elaboration of the puparium. Segment 16A first reaches a maximum size. As this puff retrogresses, segment 16B increases. As segment 16B then decreases, a large segment of the left arm (segment 13) begins to expand and ultimately becomes the largest puff seen on the entire chromosome. At the same time that segment 13 is in its maximum expansion, segment 2 on the right arm also expands. When the puffs on these two segments have again contracted, and no puffs are seen on the other chromosomes, the salivary gland rapidly degenerates so that no vestiges of the tissue remain.

During this period not only do localized areas of expansion appear in sequence as puffs, but also the

entire body of the chromosome becomes larger both in length and diameter. As this generalized expansion occurs, many newly visible bands appear. As the puffs then disappear, the chromosomes decrease rapidly in size. This decrease in size follows the extrusion of numerous spherical bodies from the chromosome. The extrusion of these bodies is particularly intense near the masses of heterochromatin in each chromosome.

We shall now describe the sequence of larval molts that precede pupation and try to relate the appearance of the various puffs to these morphological changes. Furthermore, now that very detailed data on location and sequences of puff formation are available in the four chromosomes, more basic studies on the nature of the puffing phenomena can be done.

References

[1]C. Pavan and M. E. Breuer, *Chromosoma* **7**, 371 (1955).

1.11 HETEROZYGOSITY IN PUFFS AND CHROMOSOMES OF *SCIARA OCELLARIS*

A. L. P. Perondini

The best-known cases of chromosomal polymorphisms are those involving rearrangements in chromosome sequence in several species of Diptera, especially Drosophila.[1] Very little is known, however, about the single band polymorphism shown by *Sciara ocellaris*.[2] In a strain of this species,[3] the frequencies of several of these heteromorphic bands are being studied. Our preliminary results in the analysis of 136 larvae, from 12 pair matings, for heterozygosity in ten loci suggested (1) that these polymorphic bands are transmitted as Mendelian characters, (2) there is a high degree of heterozygosity involved in this strain of flies, and (3) this heterozygosity has been maintained in laboratory cultures for more than a year.

Cytological maps of the salivary gland polytene chromosomes in late fourth instar larvae were prepared, and the pattern of puffs is now being studied. At least eight DNA puffs (as judged by their stainability and higher degree of uptake of DNA precursors) occur in this species; there are three in chromosome A, two in chromosome B, two in chromosome C, and one in the X chromosome.

Four of these DNA puffs (one in chromosome A, one in B, and two in C) undergo their puffing cycle asynchronously. 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. A few other cases of asynchronous puffs and band polymorphism are mentioned in the literature.[2,4–7] We think that the band polymorphism and the asynchronous puffs may have an important effect in speciation of these organisms.

References

[1]A. B. da Cunha, *Advan. Genet.* **7**, 93 (1965).
[2]C. W. Metz, *Genetics* **22**, 432 (1937).
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1.12 NUCLEIC ACID SYNTHESIS IN POLYTENE CHROMOSOMES OF RHYNCHOSCIARA[1]

L. C. G. Simoes

Autoradiographic studies using ^{3}H thymidine as percursor were done using larvae of Rhynchosciara in order to investigate the metabolic activity of the salivary gland chromosomes throughout the larval cycle.

It was found that three distinctive patterns of labeling can be found at any age of the larvae. Although all patterns are usually present at a given age, some are more common than others according to the larval age. We classified these patterns as: (1) uniform label (the label is regularly spread over the chromosome), in which at least three categories can be distinguished: (a) very light uniform label, (b) heavy uniform label, (c) light or heavy but with the heterochromatin more heavily labeled; (2) differential label (some well-defined bands are labeled and the rest of the bands are not); and (3) heterochromatic label (label is restricted to the heterochromatic regions of the chromosomes).

The uniform pattern is most commonly found, and in late larval life it is seen in about 90% of the chro-

mosomes. On the basis of the above labeling patterns, a tentative sequence of synthetic events in these chromosomes is being developed.

In a study of the development of the DNA puffs it was found that these puff regions begin to synthesize extra DNA several days before the puff itself appears.[2] Grain counts in chromosomes from larvae injected for a pulse of 10 min revealed that 12 days before the puff appears only 5% of the total grain counts were in the puffed region, 5 days before the puff we found 12% of the grains, 3 days before 21% of the grains, and during the puff, 50% of the whole chromosome label was in that region.

Further studies are being done in order to check DNA and RNA synthesis in the nucleolus organizer region in the X chromosome and to describe morphologically the heterochromatic regions of the four chromosomes. Studies of RNA synthesis during larval cycle and in the DNA puffs are also being done.

The present results, if compared with those previously obtained in vitro, show no substantial difference of chromosomal behavior.

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Fungal Genetics

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Radiation Mutagenesis (2.1–2.3)

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AEC-NIH Chemical Carcinogenesis (22.15)
AEC-NASA Space Biology (22.26, 22.29)

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2.1 IDENTIFICATION OF THE GENETIC ALTERATIONS IN X-RAY-INDUCED *ad-3B* MUTANTS OF *NEUROSPORA CRASSA* AT THE MOLECULAR LEVEL

H. V. Malling F. J. de Serres

Ionizing radiation is known to induce intragenic alterations, point mutations, and extragenic alterations such as chromosome deletions and rearrangements.

In *Neurospora* the frequency of point mutations increases linearly with the dose and the frequency of chromosome deletions increases as the square of the dose.[1] Despite the widespread use of x rays as a mutagen, there is no report in the literature on the identification of x-ray-induced gene mutations at the molecular level in either procaryotes or eucaryotes. This analysis is now possible with the *ad-3B* mutants of *Neurospora crassa* because (1) the degree of the specificity of chemical mutagens is known for that locus and (2) it has been shown that suppressors for mutants in this locus are very rare.

Conidia of a genetically marked two-component heterokaryon of *Neurospora crassa* were irradiated with x rays at 250 kv, 30 ma, with a 3-mm aluminum filter, at an exposure rate of 1000 r/min, total dose of 1 kr.[1] At this dose, 98% of the *ad-3B* mutants

are reparable (*ad-3B*^{R}) and result from point mutations in the *ad-3B* locus. The *ad-3B*^{R} mutants were tested for their specific revertibility after treatment with *N*-methyl-*N*′-nitro-*N*-nitroso-guanidine (MNNG), *O*-methyl-hydroxylamine (OMHA), and an acridine mustard (ICR-170). MNNG and OMHA induce only base-pair transitions in *Neurospora crassa*,[2] OMHA preferentially from GC to AT. Mutants which only revert after treatment with ICR-170 and then with a high frequency are characterized as base-pair insertions and deletions. The genetic alterations in mutants of the most common types can therefore be characterized by revertibility test with these three mutagens, and Table 2.1.1 is a guide to such a characterization. When such tests were made on the x-ray-induced mutants, the spectrum of the different types of genetic alterations given in Table 2.1.2 was obtained.

All of the 33 x-ray-induced mutants seem to be single base-pair changes; 27% of these mutants have been identified as base-pair transitions; most of these have GC at the mutant site; the leaky mutants are most likely, also, to be base-pair substitutions. The genetic alterations in those mutants which give no increase in the reversion frequency after mutagenic treatment above the spontaneous frequency have been discussed earlier, and it was concluded that a certain number of these mutants were likely to be base-pair substitutions. The frequency of base-pair substitutions is therefore at least 39% and could be as high as 66%. Base-pair insertions and deletions occur with a frequency of 34%; this type of mutations may be the result of deletion of bases from the DNA by the x irradiation.

Since x rays are very effective in inducing chromosome deletions, it was somewhat surprising that none of the 33 mutants were stable. X-ray photons can attack all parts of the DNA;[3] the ratio of the distribution of damage on the bases to deletion of the bases from the DNA to breakage of the sugar-phosphate backbone is approximately 2 : 1 : 1. Among the base pairs AT is attacked 50% more than GC. It would appear from our data that the majority of x-ray-induced point mutations result from damage of the first two types but not damage of the third type, which might be expected to give rise to stable mutants resulting from gross (base-pair) deletions.

Table 2.1.1. A Guide for Identification of the Genetic Alterations in Mutants by Means of Specific Revertibility Tests

The classification of the mutants is based on calculations of the probability that the reversion frequencies obtained in the treated series are the same as the reversion frequencies obtained in the controls. A probability below 1% is considered as significant.

+ = frequency significantly higher than that occurring spontaneously.
0 = frequency not significantly higher than that occurring spontaneously.
– = no revertants detected.

Mutation Mechanism[a]	Control	Mutagenic Treatment		
		MNNG	OMHA	ICR-170
GC → AT	+/–	+/0	+	+/0
AT → GC	+/–	+	0	+/0
+/–	+/–	0	0	+
Sign Mutations				
SP	+	0	0	0
NON	–	–	–	–

[a]See Table 2.1.2.

Table 2.1.2. The Percentages of Different Types of Genetic Alterations in a Random Sample on 33 X-Ray-Induced *ad-3B* Mutants

Base-Pair Transitions (Direction of Reverse Mutation)		Base-Pair Insertion and Deletion (+/–)	Revert Only Spontaneously (SP)	Nonrevertible (NON)	Leaky
AT → GC	GC → AT				
6	21	34	27	0	12

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2.2 ENZYME ASSAYS FOR THE *ad-3* MUTANTS OF NEUROSPORA

C. R. Fisher

Although a tremendous amount of information has been accumulated on the *ad-3* mutants of Neurospora in various types of genetic analyses, no detailed study of the enzymes controlled by the *ad-3A* and *ad-3B* loci has been undertaken. In this project an attempt is being made to develop assays for these two enzymes. The first phase of this study is the synthesis of the metabolic intermediates required by these mutants, since they are not available commercially. Initial trials have been successful, and the closely related intermediates, 5′-phosphoribosyl-5-aminoimidazole-4-(*N*-succinocarboxamide) (SAICAR) and 5′-phosphoribosyl-5-aminoimidazole-4-carboxamide (AICAR), have been isolated in gram quantities. The SAICAR thus isolated has been shown to be biologically active in a crude Neurospora enzyme preparation. It is converted from SAICAR to AICAR by the *ad-4* enzyme. The feasibility of such an assay system in Neurospora has thus been demonstrated.

Mutants of Schizosaccharomyces have been obtained which are believed to accumulate 5′-phosphoribosyl-5-aminoimidazole (AIR) and 5′-phosphoribosyl-5-aminoimidazole carboxylate (C-AIR) in large quantities. Isolation of these intermediates of the *ad-3A* and *ad-3B* loci is now in progress. When these compounds are available in sufficient quantity, they will be used in assays to obtain biochemical evidence to determine the step in purine biosynthesis blocked in each of these two groups of mutants. With these assay systems, we hope to be able to follow the enzymes through standard purification procedures and ultimately to characterize each enzyme in detail. This will then provide an approach to a number of interesting problems resulting from the genetic analyses on these mutants: (1) the nature of the proteins specified by noncomplementing mutants and by complementing mutants with nonpolarized or polarized complementation patterns, (2) the mechanisms responsible for the wide variety of leaky *ad-3* mutants, (3) the basis of phenotypic reversion, and (4) temperature-sensitive mutants.

2.3 THE EFFECT OF PHOTOREACTIVATION ON UV-INDUCED INACTIVATION OF HETEROKARYOTIC CONIDIA AND FORWARD MUTATION IN THE *ad-3* REGION OF *NEUROSPORA CRASSA*

B. J. Kilbey[1] F. J. de Serres

Ultraviolet irradiation (uv) is known to give rise to both point mutations (intragenic alterations) and chromosome aberrations (extragenic alterations).[2,3] Whether both intragenic and extragenic alterations induced by uv result from the same uv-induced premutational lesion is not known, but studies with x rays have shown that the initial events leading to

the production of *ad-3* mutations by intragenic and extragenic alteration occur at different frequencies,[4] and that these events are not subject to the same mechanisms of repair.[5]

In previous experiments[6] we have studied the effect of photoreactivation on that uv-induced premutational damage leading to intragenic alterations in a haploid wild-type strain of *Neurospora crassa*. *Ad-3* mutants which had been induced by uv in the presence and absence of photoreactivation were compared, but no evidence was found to suggest that any part of the mutant population was repaired preferentially. These results led us to conclude that in this system there is but a single type of premutational damage induced by uv that results in intragenic alteration and that repair of a fraction of this damage affects all types of mutants similarly. If there is more than one type of premutational lesion, then in terms of the mutants that they are capable of producing, both photoreactivable and nonphotoreactivable lesions are equivalent. In the present experiment we are attempting to study the effect of photoreactivation on uv-induced damage leading to extragenic alterations by using a genetically marked two-component heterokaryon in which *ad-3* mutations are produced by both intragenic and extragenic alterations.

Suspensions of conidia of the heterokaryon were treated as described previously[2] to obtain purple adenine mutants resulting from forward mutation in the *ad-3* region.

The analysis of the data on survival of the heterokaryotic conidia showed that the curves are not single-hit (as obtained with ionizing radiations)[4] but multihit. Photoreactivation gave striking increases in survival, with a dose reduction factor (DRF) of about 0.7. The dose-effect curves for forward mutation obtained with and without photoreactivation after uv treatment are shown in Fig. 2.3.1. The dose-effect curves for uv-induced forward mutation in the *ad-3* region have identical slopes (2.04 ± 0.17) with photoreactivation and (2.05 ± 0.18) in the dark. Fewer mutations were recovered after all uv exposures with photoreactivation. Covariance analysis of these data has shown a highly significant difference between these two curves ($F = 196.6$ with one and seven degrees of freedom). The DRF for mutation induction obtained with photoreactivation is about 0.6. The DRF for survival and that for mutation induction agree with previous data[2] on a haploid wild-type strain. Samples of *ad-3* mutations from each of the different

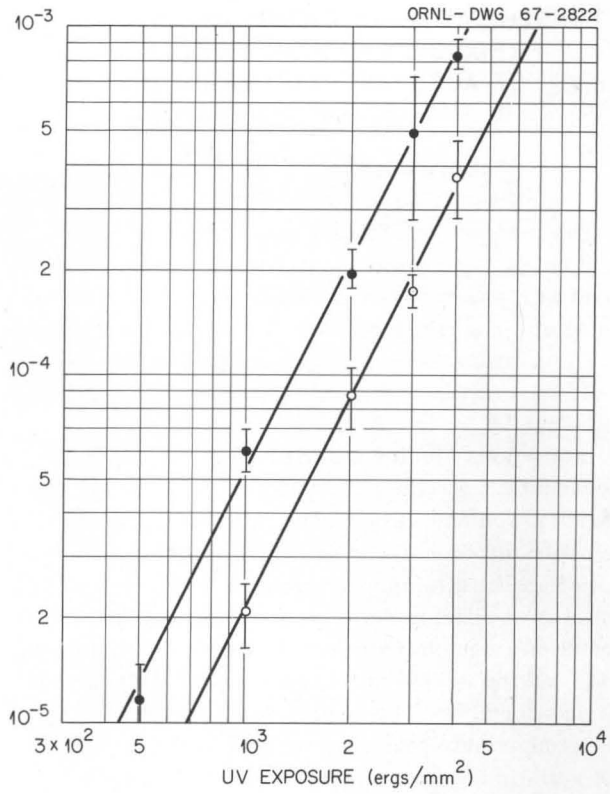

Fig. 2.3.1. Mutation-Induction Curves for Purple Adenine Mutants in the *ad-3* Region of a Genetically Marked Two-Component Heterokaryon After Treatment with UV With and Without Photoreactivation. O = photoreactived, ● = dark.

exposures (Fig. 2.3.1) have been reserved for genetic analysis, which is in progress.

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2.4 GENETIC MODIFICATION OF CROSSOVER FREQUENCY AND THE FORMATION OF ABERRANT MARKER RATIOS IN THE ASCI OF *NEUROSPORA*

J. R. Baylis, Jr. F. J. de Serres

The work of both Stadler and Towe[1] and Case and Giles[2] by random spore analysis or tetrad analysis of crosses between closely linked nonallelic or allelic markers indicates that asci in which there are departures from the expected 4 : 4 mutant-to-wild-type ratios may be expected with a frequency of about 1%.

In previous studies a series of *rg hist-3 nic-2* recombinant strains were synthesized to study recombination and interference in crosses between closely linked markers on linkage group I. These recombinant strains were crossed with three *ad-3A* strains of different wild-type origin, and the recombination frequencies in the *rg–hist-3*, the *hist-3–ad-3A*, and the *ad-3A–nic-2* regions were determined. It was found that the *rg hist-3 nic-2* strains could be grouped into four classes based on the patterns of variation in map distances and chromosome interference. The variation is related to differences in the genetic constitution of the *ad-3A* and *rg hist-3 nic-2* strains.[3–5]

Using these strains, an investigation has been made into the effects of genetically determined modifications of recombination on the frequency of asci with aberrant marker ratios. The method employed consists in screening unordered octads of ascospores obtained from crosses between the *ad-3A* strains and the *rg hist-3 nic-2* strains. A number of crosses will be compared for their differences in recombination frequencies and for the relationship of these differences to the frequency of asci with aberrant marker ratios.

From the first cross to be analyzed, 53-006-003 (*rg hist-3 nic-2*) × 38709-002-0001 (*ad-3A*), the following percentages of recombination were obtained: *rg-hist-3*, 3.45; *hist-3-ad-3A*, 1.53; *ad-3A-nic-2*, 6.10. In the 262 asci thus far examined no cases of aberrant marker ratios have been detected.

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2.5 GENETIC ANALYSES OF REVERTANTS OF *ad-3B* MUTANTS IN *NEUROSPORA CRASSA*

H. V. Malling

Suppressor mutations restore phenotypically the function affected by another mutation. The suppressor mutation can occur inside the gene (intralocal suppression) or outside the gene (extragenic suppression). The mechanism for extragenic suppression can be direct – by a change in the specificity of the translating (protein synthesizing) system – or indirect – by either relief of inhibition, by metabolic cross-feeding, or by shunting of a genetic block by opening up an alternate pathway. All three indirect mechanisms for suppression can occur in *Neurospora*, whereas suppressors which act through the translating system and which are common in yeast and bacteria have only been reported once in *Neurospora*.[1] Previous genetic analysis of revertants of *ad-3B* mutants showed no presence of extragenic suppressors, and it was concluded that indirect extragenic suppressors do not occur for the *ad-3B* mutants.[2] In the present study 264 revertants were analyzed for extragenic suppressors. The revertants were induced by nitrous acid (NA), methoxylamine (OMHA), and an acridine mustard derivative (ICR-170) in a tester set of six *ad-3B* mutants. The tester set consisted of two mutants that revert by base-pair transition from GC to AT, two mutants that revert by the base-pair transition from AT to GC, and two mutants that revert by base-pair insertion and deletion.

The revertants were made homokaryotic by crossing them with 74-IS1-30a[3] (*a, hist-2, ad-3A, ad-3B, nic-2, al-2, cot*), and the homokaryotic revertants were isolated as (*A, al-2, cot*) strains. The homokaryotic revertants were then crossed to 74-OR31-14a (*a, al-2, cot*). If reversion were due to a suppressor mutation 25% or less of the ascospores should carry the original *ad-3B* mutation. The cross is homozygous for *cot*, so that all ascospores form small tight colonies at 35°C. The development of the purple pigment in the *ad-3B* mutants is

intense at low levels of adenine (12, 5 mg/liter) and high levels of glucose. From these facts a technique was developed in which it was possible to search for the presence of one purple colony among 6000 white colonies per Petri dish (150 × 22 mm). At the 5% confidence level the frequency of purple colonies could be as high as 6 per 6000 white colonies screened from a single cross without any purple colony appearing. Since only half of the nonparental-type progeny can be discovered, suppressors which are less than 0.25 map unit from the *ad-3B* locus will not be detected by this method. None of the 264 crosses between revertants and wild-type gave purple colonies among the progeny. The greater part of genome can, therefore, be excluded as possible sites for suppressor mutation for *ad-3B* mutants.

Identification of genetic alterations by specific revertibility tests will be greatly influenced by the occurrence of suppressor mutations along with reverse mutations. The finding that *ad-3B* mutants are not genetically suppressible makes this locus unique for (1) identification of the genetic alterations at the molecular level of spontaneous and induced mutants by the specific revertibility test and (2) use of the present tester set for qualitative characterization of the mutagenic action of different compounds and treatments.[4]

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Drosophila Cytology and Genetics

D. L. Lindsley

Chromosome Behavior		Biochemical Genetics
Rhoda F. Grell	J. W. Day	E. H. Grell
D. L. Lindsley	I. Lucile Norton	
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3.1 STUDIES OF TEMPERATURE-INDUCED RECOMBINATION IN THE OOCYTE OF *DROSOPHILA MELANOGASTER*

Rhoda F. Grell J. W. Day

Introduction. – A rough coincidence between the time of DNA replication, as judged by the incorporation of ^{3}H-thymidine, and the time of crossing-over, as judged by the heat-induced alteration in exchange values, has been observed in the Drosophila oocyte.[1] Two subsequent studies have excluded the possibility that the coincidence reflects oogonial rather than meiotic events; first, genetic tests have shown that the temperature-induced crossovers are exclusively meiotic in origin;[2] second, autoradiographic studies of ovaries fixed 30 min after injection of Drosophila females with ^{3}H-thymidine show uptake of label in the 16-cell cyst (oocyte plus 15 nurse cells) located in the most anterior region of the germarium.[3]

The possibility remains that the temperature-sensitive period precedes the time of meiotic exchange, in which case temperature would precondition the younger germ cells and cause them to undergo increased recombination as oocytes. To examine this question females were heat treated (35° for 24 hr) on successive days of development from the egg stage (day 1) to the time of eclosion (day 10). Significant increases in exchange were observed only among progeny from females treated on days 6 and 7, corresponding to early pupal stages. Our studies have now focused on the period between 120 and 168 hr of development with the object of determining (1) the time of the initial appearance of the oocyte, (2) the interval before a steady-state oocyte population is reached, and (3) the relationship between these times and the temperature-sensitive period for recombination.

Methods. – Partially synchronized populations of developing females carrying suitable genetic markers for crossing-over analysis were subjected

to a 10°C increase in temperature for a 12-hr period at ages 120, 126, 132, 138, 144, 150, 156, 162, and 168 hr. The first progeny from such females, representing in each case the most mature oocytes at the time of treatment, were scored for crossing-over.

Concurrent cytological studies of the ovaries taken from pupae of age 120, 126, 132, 138, 144, 150, and 156 hr were carried out. The frequencies of one-, two-, four-, and eight-cell oogonial cysts in each age group were determined from metaphase figures. Eight-cell cysts at mitotic metaphase will become the oocyte and 15 nurse cells at the completion of the division, and such cysts were used as a measure of the oocyte population in the ovary.

Results and Conclusions. – Figure 3.1.1 presents the percentage increase in crossing-over when heat treatment is administered at different times between 120 and 168 hr. The increase reaches a maximum of ~60% at 138 hr, plateaus at this value until 156 hr, and then falls to the control level, marking the end of the temperature-sensitive period. The percentage of eight-cell cysts among total oogonial cysts at mitotic metaphase increases from ~2% at 120 hr to a maximum of ~30% at 144 hr and remains at this level.

The results indicate a good chronological correspondence between the onset of the temperature-sensitive period and the initial appearance of the oocytes as well as between the maximal temperature effect and the attainment of the steady-state oocyte population. Studies to determine the time of DNA replication in the first-formed oocytes and the relationship of this time to the temperature-sensitive period for recombination are currently under way.

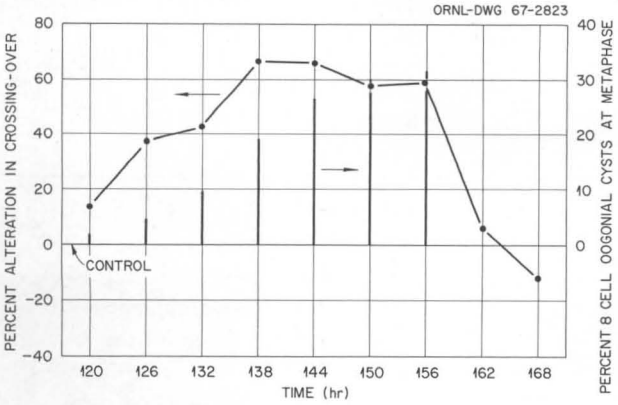

Fig. 3.1.1. Temperature-Sensitive Period for Crossing-Over and the Appearance of the Oocytes During Pupation. ●—● curve represents change in crossover frequency; bars indicate eight-cell cysts. Eight-cell oogonial cysts at metaphase used to estimate oocytes.

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3.2 MUTANTS AFFECTING MEIOSIS IN DROSOPHILA

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The meiotic divisions differ from mitosis in that homologous chromosomes associate with, recombine with (in females but not in males of Drosophila), and disjoin from one another; also, one synthetic period is followed by two rather than one nuclear division. Most descriptions of and insights into the meiotic processes in Drosophila have come from investigation of the response of both normal and rearranged chromosomes to normal meiotic control. An alternative approach has, with few notable exceptions, been virtually ignored. All biological processes are under strict genetic control, and the effects of mutations affecting different components of the genetic control system have often provided insights into the processes themselves. The few mutants known to have an effect on the meiotic divisions of Drosophila have been recovered by accident rather than by design, and we felt that it should be profitable to systematically screen a large number of chromosomes in search of meiotic mutants. Consequently, pairs of autosomes (i.e., chromosomes *2* and *3*) sampled from natural populations were made homozygous in an appropriate genotype, and crossing-over was measured in females and disjunction was measured in both sexes.

Females of constitution $In(ILR)sc^{v1}$, $y\ y^+\ sc^{v1}\ sc^+\ pn\ cv\ m\ f/y$; $2_i/2_i$; $3_i/3_i$; spa^{pol}/spa^{pol}, where 2_i and 3_i represent the ith autosomal pair = the ith genome, were crossed to $Y^SX \cdot Y^L$, $In(1)EN$, $y\ y^+\ v\ f\ B/O$; $C(4)RM$, $ci\ ey/O$ males and the progeny scored for crossing-over in the X chromosome and for nondisjunction of both the X chromosome

and chromosome *4*. Similarly, males of constitution y/Y; $2_i/2_i$; $3_i/3_i$; spa^{pol}/spa^{pol} were crossed to *y pn/y pn*; *C(4)RM, ci ey/O* females and their progenies scored for nondisjunction of the fourth chromosomes. The scope of the tests of homozygous females is shown in Table 3.2.1. We confined our testing in females to genomes in which both the second and the third chromosomes were lethal- and female-sterile-free; for this reason only about one-fourth of the genomes made homozygous were testable. Males, on the other hand, were tested if homozygotes for either the second or the third chromosome were viable and fertile; consequently, more chromosomes were tested for their effects on meiosis in males than in females. The tests used with females were more sensitive, however, and more mutants were recovered that affect meiosis in females. Eleven genomes of the 126 tested in females were selected as showing abnormal chromosome disjunction, and at least two of these (*VS51* and *VS282*) showed significant effects on crossing-over. The results with respect to disjunction and recombination for these genomes as well as for the Canton-S wild-type control are summarized in Table 3.2.2. The most commonly encountered effect is a 10- to 50-fold increase in the frequency of exceptional progeny. In some genomes the increase in nondisjunction is even more striking, and in some there appears to be a striking lack of independence in disjunctional behavior of *X* and *4*, which is evident as an excess of progeny that are simultaneously exceptional for both *X* and *4*. These genomes have been further tested for their effects on autosomal disjunction by crossing females homozygous for each genome to males carrying either attached second [*C(2L)RM*; *C(2R)RM*], or attached third chromosomes [*C(3L)RM*; *C(3R)RM*], or both. In such crosses the only viable progeny result from maternal autosomal nondisjunction followed by fertilization with appropriately nullisomic or disomic spermatozoa.

Table 3.2.1. Source and Number of Genomes Examined for Mutants Affecting Meiosis in Females

Population	Number of Genomes Sampled	Number of Genomes Viable and Female Fertile
Via Ostiense	81	18
Via Salaria	375	107
Via Tiburtina	4	1

Table 3.2.2. Disjunctional and Recombinational Data from Genomes Selected as Showing Abnormal Female Meiosis

Genome	*N*	Disjunction					Recombination				
		Exceptional for				Exceptions per Thousand	Regular Males				Map Distance
		Neither *X* nor *4*	*X* but not *4*	Not *X* but *4*	Both *X* and *4*		R_0	R_1	R_2	R_3	
Canton S	8360	8350	4	6	0	1.2	1797	1888	275	2	61.7
VO8	703	684	5	14	0	27.0	172	139	29	2	59.4
VO89	887	879	5	3	0	9.0	244	222	30	0	56.9
VS10	1207	1196	5	5	1	9.9	243	325	62	1	71.6
VS30	528	520	5	2	1	17.0	83	127	39	2	84.1
VS51	1071	1042	12	8	9	35.5	388	166	5	0	31.5
VS68	333	290	6	34	3	138.1	81	75	13	1	61.2
VS82	7	4	2	1	0	428.6	0	1	0	0	100
VS282	1528	1509	10	5	4	15.1	545	195	11	0	28.9
VS308	295	289	5	1	0	20.3	72	72	12	0	61.5
VS329	170	167	0	3	0	17.6	35	38	4	0	59.8
VS332	88	33	17	30	8	715.9	12	11	11	0	94.3

The results of these crosses are shown in Table 3.2.3. Their interpretation depends heavily upon the frequencies with which the various classes of sperm are produced, but irrespective of sperm frequencies we may conclude that many of the genomes selected on the basis of increased nondisjunction of *X* and *4* also exhibit increased nondisjunction of chromosomes *2* and *3*. *VT3* is of special interest in that it was classified as female sterile in the original tests only to prove to be fertile and a meiotic mutant in crosses to attached-autosome-bearing males.

Finally, a start has been made in determining the sequence of the autosomes in the meiotic-mutant-bearing genomes. *VS10, 51, 68, 282,* and *332* are normal, and *VO8* carries *In(3L)P* = *In(3L)63C;72E1-2* and *In(3R)Mo* = *In(3R)93D;98F2-6,* inversions commonly found in natural populations.

The data from genomes selected as having an effect on meiosis in the male are presented in Table 3.2.4. It will be noted that *VS332* is the only genome that affects meiosis in both sexes. Furthermore, the distribution of markers among exceptional females indicates that *VS332* affects the second meiotic division; the remainder of the mutants which act either in males or females but not both appear to affect the first meiotic division. From this observation we postulate that the first meiotic division is controlled by different sets of genes in the two sexes, whereas the second meiotic division is under common genetic control.

At present the effects of six of the mutants are under intensive examination by different investigators. Their position in the genome and their effect on meiosis will be studied in detail. Another line of inquiry that is in progress concerns the data from the nonmutant lines. We encountered so much variation in map distance among crosses that it was not practical to use the deviation from the mean as a criterion for selecting presumptive meiotic mutants. Since for each genome we measured crossing-over in an average of five duplicate crosses, we hope to be able to determine the relative contributions of within-genome and among-genome variability to the total observed variability and in this way to obtain some assessment of the contribution of the genotype to (i.e., the heritability of) recombination frequency. This analysis is proceeding in collaboration with Marvin Kastenbaum and Diane Smith.

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[1]Work carried out in Rome with support of NSF Senior Postdoctoral Fellowships.

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[3]Institute of Genetics, University of Rome.

Table 3.2.3. Products of Autosomal Nondisjunction from Crosses of Females Homozygous for Meiotic-Mutant-Bearing Genomes and Males Carrying Attached Autosomes

Homozygous Maternal Genome	Genotype of Father					
	C(2L)RM; C(2R)RM		*C(3L)RM; C(3R)RM*		*C(2L)RM; C(2R)RM C(3L)RM; C(3R)RM*	
	No. P_1 ♀♀	No. F_1	No. P_1 ♀♀	No. F_1	No. P_1 ♀♀	No. F_1
Canton S	70	1	80	0	83	0
VO8	75	0	62	1	91	0
VS10	51	1	101	2	44	0
VS30	51	0	71	0	45	0
VS51	108	2	105	23	484	4
VS68	38	1	39	2		
VS82	45	0	37	0		
VS282	91	5	95	42	86	1
VS308	38	0	21	1		
VS329	17	0	27	1		
VS332	92	11	94	83	219	8
VT3	40	2	65	28	91	0

Table 3.2.4. Disjunctional Data from Genomes Selected as Showing Abnormal Male Meiosis

Genome	Fourth Chromosome Constitution of Progeny	
	Regular	Exceptional
VO81	1091	269
VS8	252	197
VS332	177	29

3.3 MEIOTIC BEHAVIOR OF TANDEM ACROCENTRIC COMPOUND *X* CHROMOSOMES IN FEMALE *DROSOPHILA MELANOGASTER*

J. R. Merriam

Studies with compound *X* chromosomes are concerned with the effect of chromosome structure on crossing-over. In general, the frequency and distribution of various rank exchange tetrads is normal (similar to free *X*'s) for the majority of compound types, but significant exceptions exist.[1] It is hoped that an examination of the structural requirements for normal and exceptional behavior will yield some insight into the control of the exchange process. Past analyses of the tandem acrocentric compound *X* chromosome [= two *X* chromosomes joined in tandem sequence with a single subterminal centromere, symbolized *C(1)TA*], although severely restricted owing to difficulty in synthesizing the *TA* with heterozygous markers, suggest normal behavior.[2] The *TA* described in this report arose spontaneously bearing the long arm of the *Y* chromosome appended to the centromere as an extra arm (i.e., $TA \cdot YL$) and has been extensively analyzed in this condition.[3] Its behavior is normal with or without a free *Y* homolog. The subsequent detachment of *YL* from the compound has been effected by means of crossing-over between a free duplication bearing the y^+ allele derived from $In(ILR)sc^{vl}$) to mark its centromere (= X^P, $T(1;4)B^S \cdot y^+$) and the proximal end of the compound, that is, $TA \cdot YL + X^P,\ T(1;4)B^S \cdot y^+ \rightarrow TA \cdot y^+ + (X^P,\ T(1;4)B^S \cdot YL)$ – not recovered. The acrocentric constitution and both the presence and the detachment of *YL* of $TA \cdot YL$ and $TA \cdot y^+$ have been confirmed cytologically as well as genetically. This report is concerned with the initial results in the analysis of $TA \cdot y^+$.

The *TA* pairs in a spiral, like other tandem compounds, but generates single *X* rod chromosomes by both single and double exchanges. *TA*-bearing daughters homozygous for markers heterozygous in the mother are also produced by certain classes of double exchanges. *TA*-bearing daughters that remain + in phenotype are produced by classes of single and double exchanges and by no-exchange tetrads. The progeny of well-marked (*cv*, *v*, *f*, and *car*; all markers in coupling on the proximal arm) females without a free *Y* homolog crossed to attached *XY*, *y B* males with or without a free *Y* homolog are given in Table 3.3.1. Each of the expected single-crossover rod classes (non-*B* males) is recovered with frequencies in agreement with the standard map distances. The best estimate of the expected number of *X*-bearing eggs is the number of *Y*-bearing (or zero as in these crosses) eggs which develop as *y B* patroclinous males. In both generations the sum of *X*-bearing classes (= + females plus homozygous females

Table 3.3.1. Results of TA-Bearing Females Crossed to *XY*, *y B* Males

Phenotype of Progeny		Generation I	Generation II	
			Cross A	Cross B
y B	♂♂	225	139	339
+	♀♀	87	51	141
v	♀♀	0	1	3
v f	♀♀	0	0	2
cv v f	♀♀	1	0	0
f car	♀♀	0	0	1
B	♀♀	98	82	176
cv v f car	♂♂	24	6	17
v f car	♂♂	37	22	67
f car	♂♂	71	42	98
car	♂♂	10	3	20
+	♂♂	5	6	1

Generation I = $TA \cdot y^+/0$ ♀♀ x XY, *y B*/0 ♂♂;

Generation II, cross A = $TA \cdot y^+/0$ ♀♀ x XY, *y B*/0 ♂♂;

Generation II, cross B = $TA \cdot y^+/0$ ♀♀ x XY, *y B/FR2* ♂♂.

plus non-*B* rod males) is close to the expected number estimated from *y B* males. There were 235 viable *X*-bearing eggs compared to 225 patroclinous males in generation I, and 481 viable *X*-bearing eggs compared to 478 patroclinous males in generation II. The discrepancy between single *X* rods recovered as females (= *B* females) and as males (non-*B* males) in both generations is probably due to decreased viability of the *B* females, since it occurs with different types of fathers (with or without a free *Y* homolog). Moreover, the agreement between the number of *X*-bearing eggs expected and observed suggests that there are no major viability problems with any of the summed classes. It also suggests that there is no loss of the newly formed single *X* rods, as has been observed for single *X* rings newly generated by exchange in the tandem metacentric and the tandem ring compound *X* chromosomes.[4] On the basis of these initial results the behavior of $TA \cdot y^+$ appears to be normal, understandable, and in agreement with the behavior of other *TA's*.

There are additionally in these experiments cytological and genetic observations on two independent stabilizations of $TA \cdot YL$. The first, *C(1)s-1*, appears to be a reversed acrocentric compound *X* chromosome with *YL* appended to the centromere as an extra arm. Its genetic behavior is normal and understandable with regard to *RA's*. The second, *C(1)s-2*, appears to be an attached *X* (reversed metacentric) with *YL* appended distally on one arm. The salivary chromosomes of females bearing this attached *X* are noteworthy for the fact that asynapsis occurs over all or parts of the *X* chromosome in almost every figure. Detachments of this attached *X* should yield the first example of $YLX \cdot$, which may be useful in other connections. The genetic behavior of *C(1)s-2* is not readily understandable on any basis.

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3.4 SELECTION OF GENE MUTATIONS CAUSING THE ABSENCE OF ALCOHOL DEHYDROGENASE IN *DROSOPHILA MELANOGASTER*

E. H. Grell

Mutations causing the absence of activity of specific enzymes are in many cases easily selected in microorganisms. In Drosophila they are usually difficult to select from large numbers of normal flies. The exceptions are where the absence of an enzyme activity is accompanied by an abnormal eye color. Part of the difficulty is that Drosophila is a diploid organism with the exception of the X chromosome in the male. In the diploid there are two copies of every gene. A fly with an enzyme-negative mutation in one chromosome still contains activity for that enzyme because it has a functional gene for that enzyme in the other member of the homologous pair of chromosomes. Since it has one functional gene, it cannot be distinguished from normal flies by the usual criteria.

This problem presented by diploidy can be circumvented by first inducing a deficiency of a chromosomal segment including the desired gene. A deficiency may be selected by inducing with x rays apparent mutations of genes known to be located near the enzyme gene. In the case of Drosophila alcohol dehydrogenase (ADH), the locus that specifies the electrophoretic mobility of the enzyme was known to be near the loci of black body color and elbow wings on the second chromosome. An x-irradiated chromosome which was simultaneously deficient for these two visible mutants was found to be also lacking the *Adh* locus, which specifies the electrophoretic mobility and probably the amino acid sequence of alcohol dehydrogenase. This chromosomal deficiency [symbolized *Df(2L)64j*] is fairly large and contains many loci. Therefore it is homozygous lethal. To produce a fly without ADH another chromosome would have to be produced which lacked a functional *Adh* locus but was viable in the heterozygote with *Df(2L)64j*. To accomplish this, the following sequence of procedures was performed. Males carrying the desired *Adh* allele were injected with 0.06 *M* ethyl methanesulfonate (a potent mutagen) and mated to *B1/SM1, Cy* females. *Adh* (treated)/*SM1, Cy* sons were mated individually to *Df(2L)64j*, Pin^{Yt}/*SM1, Cy* females.

(*SM1* is a second chromosome with inversions to suppress crossing-over. The other mutant symbols are for dominant marker genes.) From each male, one offspring that was *Adh* (treated)/ *Df(2L)64j* Pin^{Y^t} was selected and crushed in a depression of a spot plate. To each depression was added 0.2 ml of the alcohol-NAD-Nitroblue tetrazolium solution used for the detection of ADH activity. After about 5 min of incubation at room temperature, the depressions containing flies with normal ADH activity had turned from the pale yellow of the original solution to a dark purple. In depressions with the rare fly with no ADH activity there was only a slight background reduction of dye and the general color was light pink. Sibs of flies that gave a negative test for ADH were mated to preserve the chromosome carrying the mutation. From the mating of *Adh-negative/SM1, Cy* females with the same kind of males, some *Adh*-negative homozygotes should be produced. They are viable and fairly vigorous under ordinary culture conditions.

In most textbooks of biochemistry a standard example of detoxication is the oxidation of alcohols. The ADH-negative mutants of *D. melanogaster* illustrate such a function of ADH. Normally the flies are very resistant to the presence of ethanol in their environment. With an initial concentration of 15% ethanol in the culture medium, flies with ADH live and breed with no obvious ill effects. ADH-negative flies placed on such medium begin to lose control of their movements within about 1 hr. They have difficulty in flying and walking and finally cannot stay on their feet. No ADH-negative fly survives for more than 24 hr on medium containing 15% ethanol.

The ethanol sensitivity of ADH-negative flies provides another method for detecting these mutations. The treatment with mutagen and the matings are the same as with the spot plate technique described above. Instead of testing one fly from each culture for ADH activity, the entire progeny is transferred to a vial containing 15% ethanol in 2% agar. There are three kinds of flies, *Df(2L)64j* Pin^{Y^t}*/SM1, Cy* and *Adh* (treated)/*SM1, Cy*, and *Adh* (treated)/*Df(2L)64j* Pin^{Y^t}. The first two types always have ADH because of Adh^F in *SM1, Cy*. The third type has ADH except when there has been an *Adh*-negative mutation in the treated chromosome. The first two have curly wings and the last has straight wings. When there has been an *Adh*-negative mutation, the straight-winged flies die on the alcoholic medium. The presence of dead flies signals the presence of a mutation. The curly-winged sibs are unaffected, and the mutation may be recovered from them.

Using these techniques five *Adh*-negative mutations have been recovered. They localize to the same locus that specifies the electrophoretic mobility. None of the ten possible heterozygotes have ADH activity. Only the ADH which catalyzes the oxidation of short-chain alcohols is affected. The activity of octanol dehydrogenase, which catalyzes the oxidation of longer-chain alcohols, is unaffected.

3.5 ANALYSIS OF A SMALL DUPLICATION

D. R. Parker

One interesting duplication, *Dp(1;4)1021*, was obtained from a group of radiation-induced fragments of a doubly marked *Y* chromosome, B^SYy^+. A small piece, originally derived from the *X* and marked with y^+, is attached to the fourth chromosome. That this duplication is small is shown by the following: this chromosome disjoins regularly from a normal fourth; it fails to induce nondisjunction of structurally heterozygous *X*'s ($In(1)49+B^{M1}$); and it lacks *Y*-derived male-fertility loci. Flies homozygous for this chromosome are fully viable and fertile; hence there is little loss of material of chromosome *4*. This would be consistent with an exchange between the tips of *YS* and of *4L*. Such marking would make this chromosome useful in looking at induced meiotic exchanges with the *X*.

That this is the case was verified by inducing a number of detachments in attached-*X/O* females homozygous for *Dp(1;4)1021* (*C(1)RM, y v bb/O; 1021*). Young females of this constitution were irradiated and mated to appropriate males, and detachments were tested for linkage of the markers of *4R*, ci^+ and ey^+. Recovered detachments were derived from exchanges in stage 7 and younger oocytes.

The first 21 detachments obtained have been analyzed. Twelve carry the markers of *4R* but not y^+, while six have y^+ but not the *4R* markers. One detachment carried both y^+ and the *4R* markers. These results are compatible with the location of y^+ on *4L*, with the single exception resulting, perhaps, from a break in *4R* distal to eyeless. In

addition, two detachments had none of the markers used.

Earlier work has shown that about 60% of all detachments recovered from young treated attached-*X/O* females have *4R* linked with the *X*. Of the remaining 40%, at most about 10% can be accounted for by exchanges with the tips of the long chromosomes. The finding that 13 out of 21 detachments carry *4R* markers is compatible with the idea that *Dp(1;4)1021* shows a similar exchange distribution to the normal fourth, and that one may be able to account for a large number of detachments in the earlier experiment arising by exchange with *4L*.

Questions of *X-4* exchange during meiosis in the female are being reexamined, and this chromosome will be useful especially in the case of stage 14 detachments, where the usual tests have failed to account for fully 50% of the exchanges that give rise to detachments.

3.6 TRANSLOCATIONS AND RECOMBINATION

J. H. Williamson

Homozygous *2;3* translocations decrease recombination of *X* chromosome markers in *Drosophila melanogaster*.[1] This observation could be the result of homozygosity for position effects of specific breakpoints of the translocations or of disrupted chromosome continuity, since *2;3* translocation homozygotes carry no large autosome of normal sequence. *X* chromosome recombination was measured in females heterozygous for two *2;3* translocations in an attempt to differentiate between these two hypotheses. Of 18 tested combinations of two *2;3* translocations, none decreased recombination of *X* chromosome markers; 12 combinations significantly increased recombination in *X*.

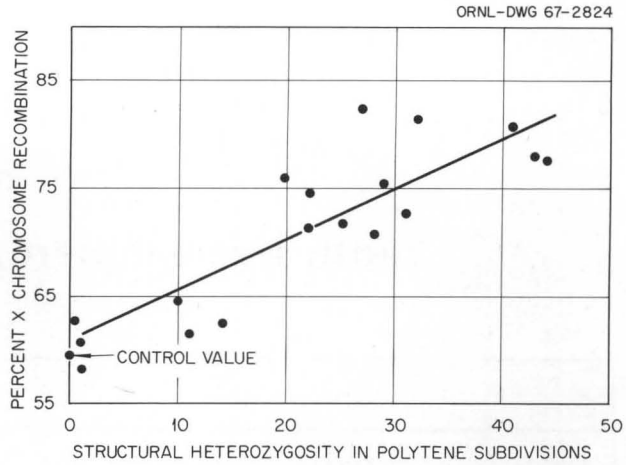

Fig. 3.6.1. X Chromosome Recombination in Females Heterozygous for Two *2;3* Translocations.

Double-translocation heterozygotes possess varying amounts of structural heterozygosity, that is, the distance between the second-chromosome breakpoints plus the distance between the third-chromosome breakpoints in the two translocations. When this dissimilarity is measured in polytene subdivisions, *X* chromosome recombination can be shown to increase as the amount of structural heterozygosity increases (Fig. 3.6.1).

The three combinations of translocations which had little or no structural heterozygosity indicate that the effects of homozygous translocations on *X* chromosome recombination are due to homozygosity for position effects at the breakpoints and not due to the absence of the large autosomes in normal sequence.

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Yeast, Hymenoptera, and Phage Genetics

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AEC-NASA Space Biology (22.27, 22.28)

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[d]Research associate
[e]Loanee

4.1 REVERSION RATE STUDIES OF THE tr_{1-1} MUTANT

R. C. von Borstel C. M. Steinberg

Introduction. – In a preliminary report[1] we presented evidence that the tr_{1-1} mutant had approximately the same intralocus reversion rate during mitosis and meiosis. We also presented data that indicated that there is a higher rate of mutation to suppressors of tr_{1-1} during meiosis than during mitosis. We attribute the lack of a meiotic effect to the original tr_{1-1} mutation being the consequence of a substitution of one of the bases of DNA.[2] We attribute the presence of a meiotic effect in the suppressors to their being the consequence of a deletion or addition of bases.[3] The results support the belief that these suppressors are mutations in the genes that encode sRNA.[1,4] Because they suppress mutants in a number of genes in nonrelated metabolic pathways, these suppressors are called supersuppressors.[5]

During the measurements of the reversion rates of the tr_{1-1} mutant, we encountered a phenomenon of continuous appearance of revertant colonies in cultures where growth had been limited by tryptophan deprivation.[6] By studying a number of different strains of haploids and diploids bearing the tr_{1-1} mutant we were able to obtain better estimates of the reversion rates than before. Our new estimates fully support our previous claim of a lack of a meiotic effect for the tr_1 locus itself and the presence of a large meiotic effect for the suppressors of the tr_{1-1} mutant.

Results. – Three haploid strains carrying the tr_{1-1} mutation and two diploid strains homoallelic for the tr_{1-1} mutation were tested for their reversion rates during mitosis by the liquid culture method.[6] The cumulative appearance of revertants is shown in Fig. 4.1.1. It can be seen that the daily accumulation of revertants varies from strain to strain and that a plateau is never achieved with

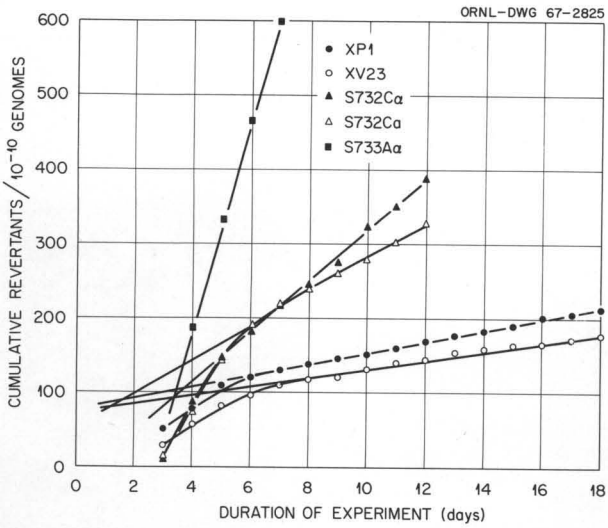

Fig. 4.1.1. The Accumulation of Revertants from Tryptophan Dependence to Tryptophan Independence in Various Strains of Saccharomyces Which Carry the tr_{1-1} Mutation. Diploid: ●, O; Haploid: ▲, Δ, ■.

any strain. We assume that after the first few days the appearance of new revertants comes from cell turnover. Therefore the real mutation rate can be estimated by extrapolating from the long linear rise back toward the vertical axis. When this is done, all curves intersect at between 90 and 100×10^{-10} reversions per genome per generation.

The proportions of intralocus and supersuppressor mutations for several strains are shown in Table 4.1.1. It can be seen that the mutation rates during mitosis are quite different for the locus and the supersuppressors, but the proportions do not vary appreciably from strain to strain in either the haploids or the diploids.

Consolidating the data in Fig. 4.1.1 and Table 4.1.1, the mutation rate for the tr_1 locus during mitosis is about 90×10^{-10}; the mutation rate of the supersuppressor loci during mitosis is about 9×10^{-10}.

The mutation rates occurring during meiosis for the tr_1 locus and the supersuppressor loci are presented in Table 4.1.2. It can be seen that the tr_1 locus mutation rate is essentially the same as that for the locus during mitosis. The mutation rate for the supersuppressor loci is about 50 times greater during meiosis than during mitosis. All cultures of cells used for sporulation were tested to estimate the proportion of mutant cells already present in the vegetative culture. The estimate of the reversion rates during meiosis entails a correction for mutations which arise during vegetative growth.[7] Since the correction is large the value is occasionally negative (Table 4.1.2).

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Table 4.1.1. Proportion of Revertants During Mitosis Tested for Mutations in the tr_1 Locus or in Supersuppressor Loci

Strain	Locus Mutants/Total	Supersuppressor Mutants/Total
Diploids		
XP1 (early revertants)	89/100	11/100
XP1 (late revertants)	86/98	12/98
XV23	65/67	2/67
Haploids		
S732Cα	88/98	10/98
S732Ca	82/90	8/90
S733A	85/94	9/94
Total	590/650 = 0.908	60/650 = 0.092

Table 4.1.2. Reversion Rate of the XP1 Strain During Meiosis

Replicate	tr_1 Locus	Supersuppressor
	$\times 10^{-10}$	$\times 10^{-10}$
1	−73	572
2	269	670
3	252	150
4	74.7	233
5	85.7	668
6	−106	465
7	144	510
8	174	364
Average	115	454
Medium	103 (average 5 and 7)	487 (average 6 and 7)

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4.2 ELECTROPHORETIC PATTERNS OF XANTHINE DEHYDROGENASE IN TWO SPECIES OF HABROBRACON

R. H. Smith L. R. Valcovic

Habrobracon juglandis and *Habrobracon serinopae* are sibling species. *H. serinopae* is native to India and *H. juglandis* has been found in a number of localities throughout North America. Mating and insemination occur in crosses between the two species but not fertilization. The Indian species has a higher oviposition rate and lives about three times as long as *H. juglandis*. Another distinguishing characteristic is the rate of uric acid accumulation throughout the adult life-span. The uric acid is seen as disk-shaped deposits within the abdomen and is associated with the fat body. *H. serinopae* accumulates urate at a slower rate than *H. juglandis*, but by the time both species die the sizes of the urate deposits are equal. Clark[1] believes there is a direct correlation between life-span and rate of urate deposition.

Xanthine dehydrogenase oxidizes hypoxanthine and xanthine to uric acid. Although there is no difference in activity of this enzyme between these two species, there is a difference in activity between diploid females and haploid males within each species.[2]

In the present study, mobility of xanthine dehydrogenase from *H. serinopae* was found to be 6% faster than from *H. juglandis* when homogenates of whole wasps were electrophoresed on polyacrylamide gel. There is one major band, and no qualitative differences were found among larvae, pupae, and sexes within each species.

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4.3 THE INTERSEX LOCUS IN HABROBRACON

M. B. Baird R. C. von Borstel

Introduction. – A mutation to an intersex condition, *ix*, has occurred on four separate occasions. The data of three of these occurrences have been published.[1] Unfortunately, the mother was dead in each case so that it was not possible to preserve the mutant strain because the male with the intersex gene has a female abdomen and male outlook. Fortunately, either the orange, *o*, or the ivory, o^i, an allele of the orange locus, was present in each case in a heterozygous condition. Thus, *ix* was found to be linked to *o*, and by summing the data from the four occurrences, a map distance of 26 centimorgans was established. The intersex mutation has recently recurred, the mother was found to be alive, she was mated, and the mutated locus was preserved.

Results and Discussion. – The map distances reported previously were from very few data. The first experiments performed with the new occurrence of the intersex mutation were to obtain better linkage values. These data, from three separate experiments, two with *o* and one with o^i, are shown in Table 4.3.1. It is evident that the published linkage value of *ix* and *o* is essentially correct.

The mutant short antennae, *sa*, in Habrobracon is undoubtedly at the same locus as the lost gynoid mutant *gy*. Recombination data showed that it is located in the same position,[2] and it has the same appearance as the gynoid mutant strain. We shall refer to it as the gynoid mutant, since it is more descriptive of the actual mutant condition. The males, though behaving as males, have a completely female appearance except for the genitalia. It is a fully viable mutant stock.

From the cross of *gy* males to +/*ix* females, two types of females were obtained. When these were bred as virgins, half of them (+/*ix*, *gy*/+) produced haploid progeny of the genetic constitution *ix*, *gy* (Table 4.3.2).

Table 4.3.2. Offspring of Virgin Habrobracon Females of the Genotype +/*ix*, *gy*/+

Phenotype	Genotype	Number	Percent of Total
Males	+, +	164	25.2
Gynoids	+, *gy*	141	21.7
Intersex	*ix*, +	170	26.1
Gynoid intersex	*ix*, *gy*	176	27.0

In every respect these gynoid intersexes had the external phenotype of females. The ovaries and uterine sacs of the gynoid intersexes were better developed than in the intersexes. Their behavior is very weakly male or even neuter when compared with the mild to moderate male attitude of the intersexes.

References

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[2] R. C. von Borstel, unpublished data.

Table 4.3.1. Linkage Tests of Virgin Habrobracon Females of the Genotype $\frac{o\ +}{+\ ix}$ or $\frac{o^i\ +}{+\ ix}$

Phenotype	Genotype	Experiment I (*o*)	Experiment II (*o*)	Experiment III (o^i)	Total	Percent of Total
Black-eyed male	+ +	93	49	36	178	0.116
Black-eyed intersex	+ *ix*	322	169	85	576	0.376
Orange- or ivory-eyed male	*o* + o^i +	323	169	62	554	0.362
Orange- or ivory-eyed intersex	*o ix* o^i *ix*	113	71	40	224	0.146

4.4 NATIVE DNA WITH PRIMING ACTIVITY IN AMPHIBIAN CHROMOSOMES

R. C. von Borstel O. L. Miller, Jr.[1]
F. J. Bollum[2]

Introduction. – Fixed tissue preparations can be used to prime DNA synthesis.[3] The standard practice is to fix the tissues in 70% alcohol followed by denaturation of the DNA in the tissue by treating it for 30 min at room temperature with 0.01 to 0.1 *N* HCl. Without prior denaturation of the DNA, the calf thymus DNA polymerase system cannot synthesize a new strand[4] on DNA from many sources, including bacteria, phage, protozoa, insects, teleosts, and mammals.

Results. – In the course of an attempt to find out if DNA steresis[5] (paling) by a microbeam of ultraviolet radiation was in fact a manifestation of denaturation of DNA in living cells, native DNA with priming activity was found in cardiac epithelial cells of *Triturus viridescens*. Metaphase chromosomes of these cells had been irradiated with ultraviolet light, and the cells were immediately fixed in 70% alcohol. The nuclei of all the cells, including those in metaphase, whether irradiated or not, were able to incorporate the labeled triphosphate deoxyribonucleosides. The irradiated regions of the metaphase chromosomes showed no particular enhancement of uptake of the labeled compounds,[6] but a slight effect may have been masked by the general uptake of the labeled compounds.

With the wealth of information available on amphibian lampbrush chromosomes, it seemed only natural to attempt to observe their priming ability with and without a pretreatment at a low pH. It was found that fully extended lampbrush chromosomes of *Triturus pyrrhogaster* are able to act as primer for DNA synthesis without prior treatment at a low pH. When so treated, there is no enhancement of their priming ability. In later stages of oocyte development, after the chromosomes are somewhat contracted, they are still able to prime DNA synthesis without prior treatment in HCl, but the treatment at a low pH does enhance the priming activity.

Discussion. – It seems evident that amphibian chromosomes do indeed have the ability to prime DNA synthesis without prior denaturation. The first reaction to an unexpected observation is to search for the possible artifacts. It is possible that the DNA is somehow denatured during the process of fixation. Certainly this occasionally is true for cell smears if they are allowed to fix by air drying.[3] But drying was carefully avoided with these tissues, and it seems unlikely that any proteins with denaturing activity would still be active after immersion in alcohol. Observation of this phenomenon on two separate tissues of two species of Triturus *and nowhere else* gives additional weight to the phenomenon not being an artifact.

The simplest interpretation is that some of the DNA in the chromosomes of salamander cells is essentially single-stranded. Thus it can act as a primer for DNA synthesis in the calf thymus polymerase system.

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[1]Cell Growth and Differentiation section.

[2]Present address: Department of Biochemistry, University of Kentucky Medical Center, Lexington, Ky.

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[6]We are indebted to Professor W. Bloom for his help in the irradiation and preparation of the tissues used in the preliminary stages of this study.

4.5 GROWTH INHIBITION WITHOUT INHIBITION OF DIFFERENTIATION BY RIBONUCLEASE IN CHICK EMBRYOS

S. K. Brahma[1] R. C. von Borstel

Introduction. – It has long been known that ribonuclease reduces the rate of cell division.[2,3] Apparently it can enter directly into living cells and digest ribonucleic acid in situ.[4] Cytochemical evidence has been presented that indicates that digested RNA in living cells may be an artifact from lack of destruction of ribonuclease by the usual fixatives with subsequent destruction of RNA after fixation and before staining.[5] This cannot always be true, because it is known that nucleoli disappear in living cells when the cells are treated by ribonuclease.[6]

Taking the lead from a report that ribonuclease inhibits cell division without inhibiting differentiation in chick embryos,[7] we decided to repeat the

essentials of the original experiments and look further into the possible mechanisms of the dissociation of growth from differentiation.

Results. – The results are shown in Table 4.5.1. In series A through C the eggs were injected with ribonuclease (five times crystallized protease- and salt-free, Sigma) either in 0.01 *M* PO_4 buffer at pH 7.1 or in 123 *mM* NaCl. They were left overnight at room temperature and then incubated for 72 hr at 37.5°C. Typical treated embryos are shown in Fig. 4.5.1*a*, and the control at the same magnification is shown in Fig. 4.5.1*b*. It is clear that growth has been impaired without inhibition of the differentiation process.

In series D the eggs were incubated for 24 hr, injected, left overnight at room temperature, and then incubated for nearly 48 hr. In series E the eggs were incubated for 48 hr, injected, left overnight at room temperature, and then incubated for nearly 26 hr. A treated embryo from the E series is shown in Fig. 4.5.2*a*, and its control is shown in Fig. 4.5.2*b*. Again, impairment of growth can be seen (though not as marked as in the embryos from the A series) with out attendant impairment of the differentiation processes. In all cases the controls were injected with either the buffer or with 123 *mM* NaCl in the same amount, and they were treated in the same ways.

Discussion. – It seems clear that ribonuclease inhibits growth by inhibiting cell division, and yet the processes controlling differentiation remain unimpaired. The independence of these two phenomena has been known for a number of years to be true for plant cells and tissues.[8] The same principle has been postulated for embryogenesis of animals largely from the studies on sea urchin eggs, where blastomeres separated at the four-cell stage develop into embryos approximately one-fourth the normal size.[9]

The straightforward interpretation of ribonuclease inhibition of division must be that the ribonuclease enters the cells, breaks down messenger RNA, and thus inhibits protein synthesis.[6] This would inhibit cell division and growth. If ribonuclease enters cells, then either the factors controlling differentiation are already present as proteins or as very well-protected ribonucleic acids. Alternatively, if the factors controlling differentiation are indeed ribonucleic acids, but not well protected, then the information necessary for development must be present in redundant sets or in RNA that is being continuously synthesized.

References

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Table 4.5.1. Effect of Ribonuclease on Growth of Chick Embryos

Series	Concentration Used (mg)	Number Injected Experimental	Number Survived	Stunted Growth (%)	Number Injected Control	Number Survived	Normal Growth (%)
A	0.5/0.1 ml	19	10	60	20	17	71
B	1.0/0.1 ml	18	15	13	8	8	50
C	1.5/0.15 ml	12	12	33	13	13	54
D	0.25/0.1 ml	66	34	33	20	13	92
E	0.5/0.1 ml	55	48	46	19	19	68

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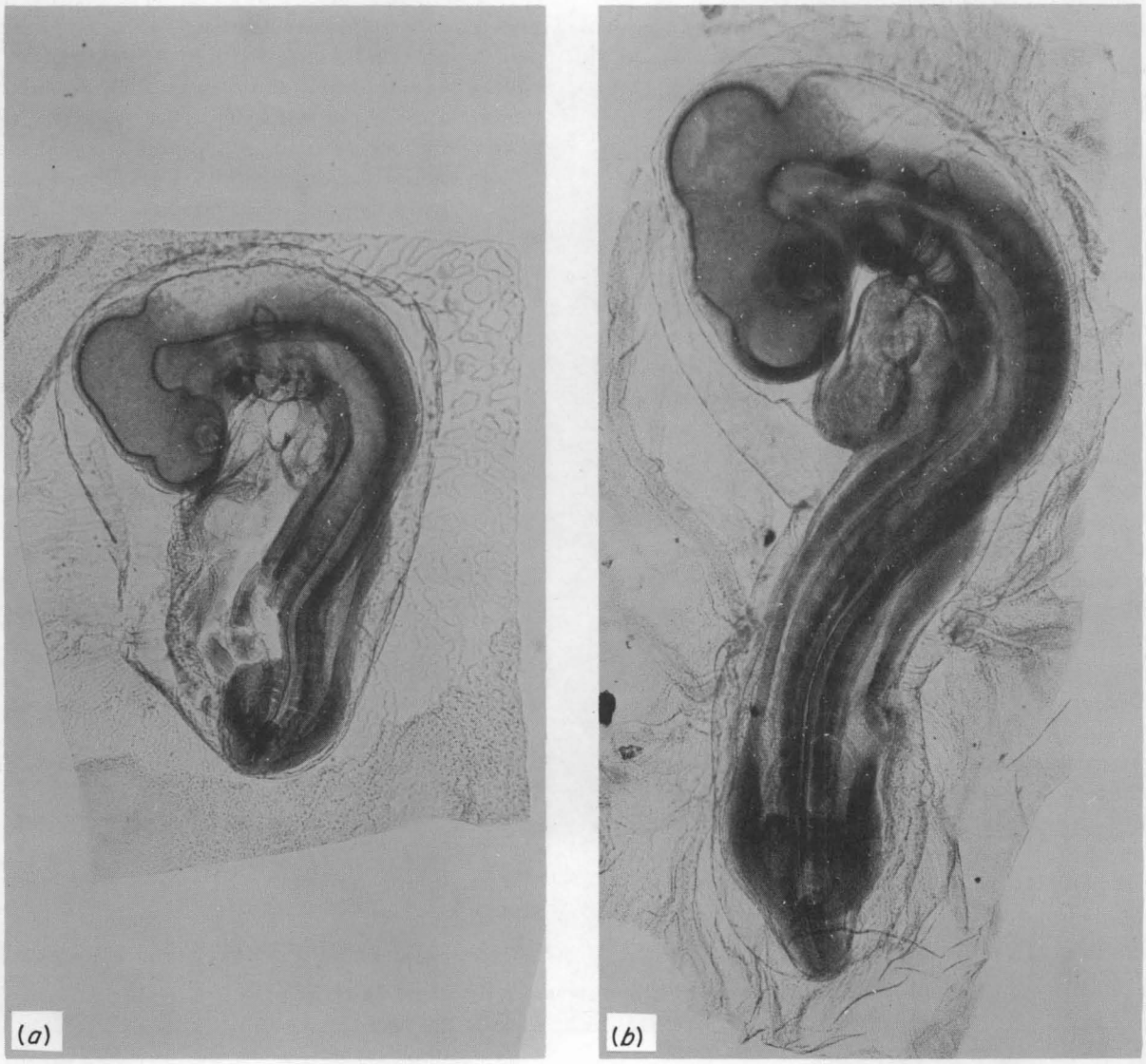

Fig. 4.5.1. (*a*) Embryo from Egg Injected with Ribonuclease Solution and Then Incubated for 72 hr; (*b*) Control for Embryo Shown in Fig. 4.5.1*a*, Same Magnification.

PHOTO 86863

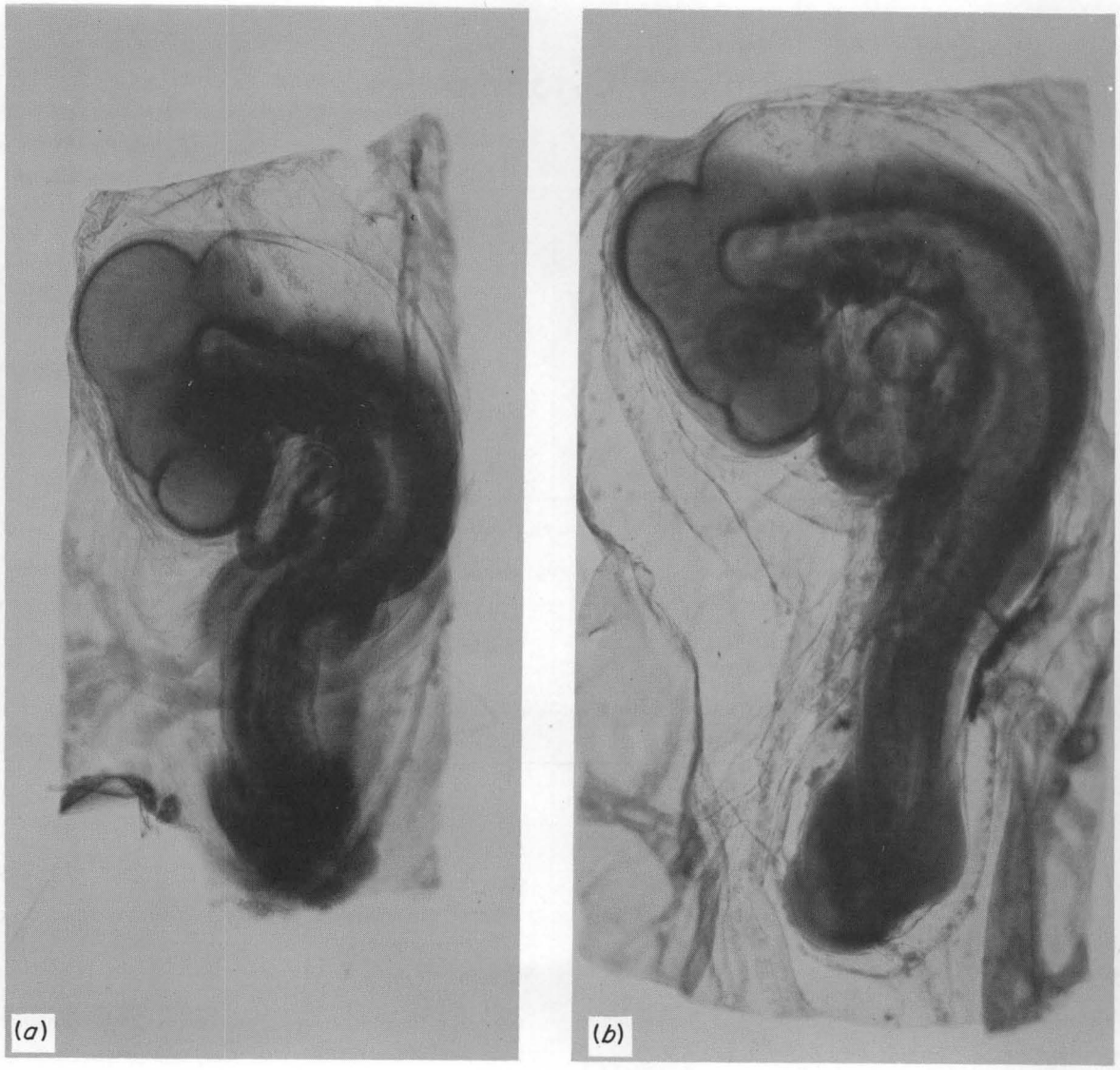

Fig. 4.5.2. (*a*) Embryo from Egg Incubated for 48 hr, Injected with Ribonuclease Solution, Left Overnight at Room Temperature, and Then Incubated for 26 hr; (*b*) Control for Embryo Shown in Fig. 4.5.2*a*, Same Magnification.

Human Cytogenetics

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5.1 CHROMOSOME ABERRATION YIELD AS A FUNCTION OF TIME OF FIXATION OF HUMAN LEUKOCYTE CULTURES

M. A Bender

Introduction. – Several groups[1,2] have noted that the yield of chromosome aberrations in short-term cultures of irradiated human leukocytes shows significant variation, depending on the time the cultures are fixed. A decrease in aberration yield might be expected if the cultures have been incubated long enough to contain a significant proportion of second divisions, because acentric fragments and some of the multiple-break aberrations would be lost at the first mitosis. Buckton and Pike[2] have taken observed decreases in aberration yield from 48 to 72 hr in their cultures as evidence of a high proportion of second divisions in cultures fixed later than 48 hr and have naively asserted that this is true for *all* leukocyte cultures. A similar assumption has been made by Sasaki and Norman.[3]

Several checks on whether or not second divisions are actually present in a sample are possible, for example, ^{3}H-thymidine labeling, or checking the frequency of cells having multiple-break aberrations but lacking the expected acentric fragments. Evidence from such checks (and also various other observations) suggests that the aberration yield in cultures of irradiated leukocytes varies with time in culture before the appearance of a significant proportion of second divisions. Although there are always some two-break aberrations lacking fragments, their frequency rises from less than 10% to over 30% at the time the second divisions start appearing in the sample.

The present experiments are part of a series designed to elucidate the variation in aberration yield as a function of time in culture in the leukocyte system (and also the effects on this relation of temperature, culture technique, etc.).

Materials and Methods. – The cell cultures were obtained from leukocytes from a single normal donor. The whole, heparinized blood samples were given a 150-r exposure to x rays. Four-milliliter aliquots were centrifuged, and the buffy coat was dispersed in culture medium containing nonhomologous human serum and 5% phytohemagglutinin (Difco). All cultures were incubated at 36°C. The cultures were fixed at various times, following a 5-hr pretreatment with colchicine.

Results. – The results of two experiments are given in Table 5.1.1. These two experiments overlapped for two fixation times (72 and 78 hr). The results of the experiments for the overlapping times of fixation were not different, so the two experiments were pooled to yield the graph shown as Fig. 5.1.1. The frequency with which two-break aberrations lacked the expected acentric fragment increases sharply between 60 and 66 hr, indicating that a significant number of second (or later) divisions are included in samples from 66 hr on. The fall in the yields of both single- and two-break aberrations, however, has clearly started much earlier. On the other hand, there appears to be little if any decrease in aberration yield for many hours *after* the appearance of an appreciable proportion of second divisions.

Discussion. – The simplest hypothesis which will explain both the change in aberration yields *before* the appearance of second divisions and the failure of the aberration yields to decrease further *after* their appearance is that there are at least two leukocyte populations, which differ both in their aberration yields and in the time they enter mitosis. If the cells which enter mitosis earliest are more sensitive, then the decrease during the "first division" interval is explained. If this same population also reaches the second division earlier than the slower, less sensitive population, the apparent cyclic nature of the aberration yield in relation to time in culture (Fig. 5.1.1) might be expected.

It is obvious that the time scale for these cultures is not absolute. Many things, including radiation exposure, for example, from incorporated iso-

Table 5.1.1. Frequencies of Chromosome Aberrations in Cultures of X-Irradiated Human Leukocytes Fixed After Various Incubation Times

Time (hr)	Cells Scored	Deletions	Rings and Dicentrics	Rings and Dicentrics Without Fragments	Percent Deletions[a]	Percent Rings and Dicentrics	Percent Rings and Dicentrics Without Fragments
				Experiment 1			
48	400	80	86	2	20.0 ± 2.2	21.5 ± 2.3	2.3
54	306	47	72	5	15.4 ± 2.2	23.5 ± 2.8	6.9
60	320	35	58[b]	5	10.9 ± 1.8	18.1 ± 2.4	8.9
66	400	66	46	17	16.5 ± 2.0	11.5 ± 1.7	37.0
72	400	67	59	21	16.8 ± 2.0	14.8 ± 1.9	35.6
78	400	76	71	23	19.0 ± 2.2	17.8 ± 2.1	32.4
				Experiment 2			
72	200	39	24	6	19.5 ± 3.1	12.0 ± 2.4	25.0
78	200	28	31	14	14.0 ± 2.6	15.5 ± 2.8	45.2
84	200	25	28	16	12.5 ± 2.5	14.0 ± 2.6	57.1
90	200	25	15	11	12.5 ± 2.5	7.5 ± 1.9	73.3
96	200	23	14	9	11.5 ± 2.4	7.0 ± 1.9	64.3

[a] ± Poisson variance.

[b] One tricentric counted as two dicentrics.

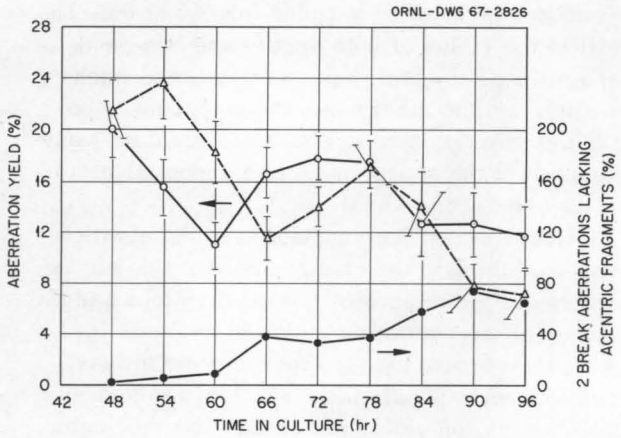

Fig. 5.1.1 Frequencies of Single- (O) and Two-Break Aberrations (△) and of Two-Break Aberrations Lacking the Expected Acentric Fragments (●) as a Function of Time in Culture: All samples given a 150-r x-ray exposure before culture.

topes such as tritium, can be expected to change the rate at which the leukocytes transform and enter mitosis. Preliminary results of other experiments show that (not unexpectedly) differences in culture technique and even small differences in incubation temperature also affect this rate. It is thus important to take such variables into account when interpreting aberration yields determined with the human leukocyte system, particularly when evaluating apparent differences obtained by different laboratories.

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5.2 HIGH CHROMOSOME BREAKAGE RATES IN LEUKOCYTES OF A NORMAL FEMALE

P. Carolyn Gooch C. L. Fischer[1]

A female volunteer blood donor, participating in an experiment as a control, was found to have a high number of chromosome-type breaks in untreated cultures of her leukocytes. These aberrations appeared always to be in the same position in the same chromosome and were seen with frequencies up to 21.6% in various untreated samples.

In blood samples obtained approximately 1 year and $1\frac{1}{2}$ years after the initial observations, the same type of breakage was again observed. Karyotypes constructed from the original and subsequent blood samples showed most breaks were in chromosomes placed in the number 10 position.

Analyses of the chromosomes of members of the subject's family are in progress. Preliminary results indicate the same type of breakage is present in the leukocytes of her brother and possibly her mother as well.

Discussion. – Dekaban[2] has observed the same type of breakage in the number 9 chromosome of an apparently normal mother of a leprechaun. The woman had, however, previously been given a course of dermal x-ray therapy. Lubs has seen a similar high breakage frequency in the number 8 chromosome of a grossly abnormal infant.[3] Emerit has also seen such breakage in the number 8 chromosome in a child with congenital heart disease.[4] Our subject has no clinical history of irradiation or disease to account for the breakage seen.

The present case demonstrates that such a peculiar high aberration frequency in a specific chromosome is not necessarily related either to radiation therapy or a familial history of physical abnormalities and may, in fact, be an inherited phenomenon.

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[3]H. A. Lubs, personal communication.

[4]Ingrid Emerit, personal communication.

5.3 THE FATE OF ULTRAVIOLET-LIGHT-INDUCED THYMINE-CONTAINING DIMERS IN HUMAN CELLS GROWN IN VITRO

J. D. Regan J. E. Trosko[1]

Introduction. – Many bacterial strains which are resistant to killing by ultraviolet radiation have the ability to excise ultraviolet-induced pyrimidine dimers from their DNA. It has been shown that some mammalian cell lines (Chinese hamster and murine "L" cells) do not excise pyrimidine dimers (as measured by the dimerized thymine/thymine ratio appearing in the TCA-soluble fraction after uv).

We have recently found evidence for preferential removal of thymine dimers in three human cell lines (RA, RAX10, and HeLa).

Results. – Forty-eight hours after uv irradiation, about 40 to 50% of the radiation-induced thymine dimers disappear from the TCA-insoluble fraction (Table 5.3.1). Furthermore, there is a concomitant appearance of dimers in the TCA-soluble fraction. Dose in the range 50 to 150 ergs/mm^2 had no significant effect on the rate of decrease of the dimerized thymine/thymine ratio in the insoluble fraction (Fig. 5.3.1).

Discussion. – If the shift in dimer ratios seen in these experiments was due wholly to ^{3}H-thymidine reutilization, then it is necessary to postulate that Chinese hamster and murine cells in vitro do not conserve label, whereas human cells in vitro do. This seems an unlikely postulate in view of the metabolic uniformity exhibited by most mammalian cell lines. Under the best conditions, however, we have been unable to achieve greater than 50% dimer removal in human cells.

At present we are examining the possible existence of more efficient dimer excision mechanisms in established cell lines other than human and rodent.

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[1]Cytology and Genetics section.

Table 5.3.1. Distribution of Radioactivity Between Thymine and Pyrimidine Dimer $\widehat{XT}/T$[a] Regions in TCA-Soluble and -Insoluble Fractions of Ultraviolet-Irradiated[b] Human Cells[c]

Cell Line	0 hr		24 hr		48 hr	
	$\widehat{XT}/T$ Insoluble	$\widehat{XT}/T$ Soluble	$\widehat{XT}/T$ Insoluble	$\widehat{XT}/T$ Soluble	$\widehat{XT}/T$ Insoluble	$\widehat{XT}/T$ Soluble
RA	420/1,148,000	0/4300	170/717,500	90/6130	110/659,200	340/23,012
	0.037%	0.0%	0.024%	1.45%	0.017%	1.48%
RAX10	109/310,300	0/5200	120/449,000	44/1900	68/325,200	105/5190
	0.035%	0.0%	0.027%	2.31%	0.021%	2.01%
HeLa	160/460,000	0/3300	76/342,800	15/1120	62/255,000	38/2676
	0.035%	0.0%	0.022%	1.34%	0.024%	1.38%

[a]Counts/min above background (figures are the average of two 10-min counts). No attempt was made to quantitate the amount of radioactivity put on the chromatography paper.

[b]50 ergs/mm^2 of 2650 A uv at 5 ergs mm^{-2} sec^{-1}.

[c]Cell cycle time for these cells was approximately 30 hr.

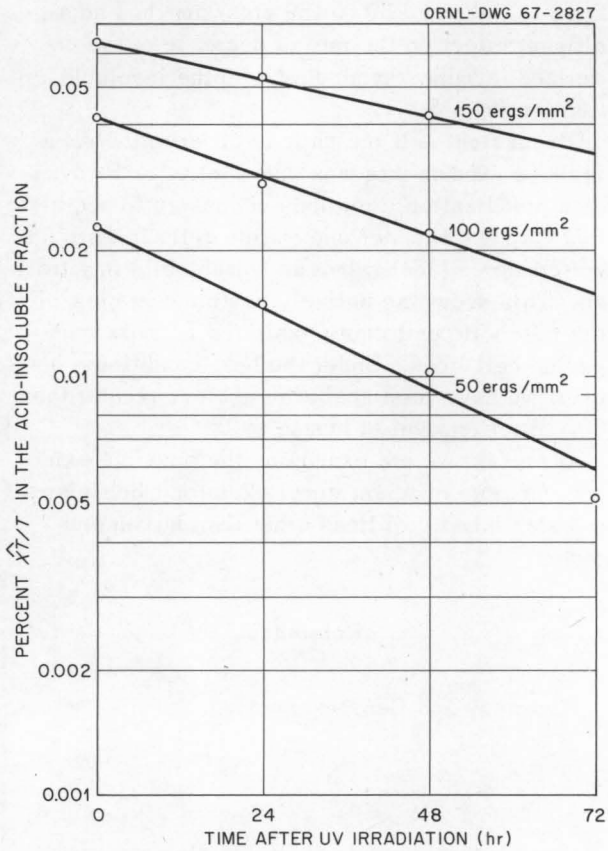

Fig. 5.3.1. UV Dose Effect on Disappearance of Thymine-Containing Dimers from TCA-Insoluble Fraction of RA Cells. Figures have been corrected for radioactivity in dimer region of the nonirradiated cells (0.009%).

Cell Growth and Differentiation

Tuneo Yamada

Control of Cell Differentiation (6.1–6.4)

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D. S. McDevitt[a]
Artur Jurand[b]
S. P. Modak[b]
Egidio Puccia[b]
Lola M. Kyte
D. H. Reese
Marion E. Roesel
Isaura Meza[b]
Georgia Morris[c]

Chromosome Ultrastructure (6.5–6.7)

O. L. Miller, Jr.
George Gassner III[d]
Barbara R. Beatty

Vitellogenesis (6.8–6.11)

R. A. Wallace
J. N. Dumont
Marilyn S. Kerr[e]
D. W. Jared
Susan L. Walker[c]

Biochemistry of Cell Differentiation (6.12–6.17)

John Papaconstantinou
R. E. Shore[d]
J. W. Carr
Anna M. Henley
Emilia M. Julku
Winnie G. Palmer[f]
Edward Stavnezer[g]
J. A. Stewart[h]
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[a]Biology Division postdoctoral investigator
[b]Visiting investigator from abroad
[c]Student trainee
[d]Research associate
[e]USPHS Postdoctoral Fellow
[f]ORAU Predoctoral Fellow
[g]Temporary
[h]USPHS Predoctoral Fellow

6.1 CELL GENERATION TIME IN VARIOUS STAGES OF LENS REGENERATION

Sara Eisenberg[1]
Tuneo Yamada

In continuation of the work reported earlier on the cell cycle of the proliferating cell population of Wolffian lens regeneration in the adult newt,[2] the average generation time was calculated for the proliferating zone of lens regenerates at various stages. For this purpose, the synthetic index was determined for such a cell population of individual regenerates, and the average cell generation time was computed using the value of *S* period of 15-day lens regenerate reported earlier.[2] In other words, calculation was made on the assumption that the *S* period is the same at all stages of regeneration in the proliferating zone. Thus obtained results are summarized in Table 6.1.1. A gradual decrease of the values with the progress of regeneration from stage I to X is indicated.

It has been shown that the proliferating zone of lens regenerates changes its synthetic properties with the progress of regeneration:[3] Incorporation of ^{3}H-leucine[4] and ^{3}H-uridine[5] increases with the progress of regeneration in the proliferating zone;

Table 6.1.1. The Mean Cell Generation Time of the Proliferating Zone of Lens Regenerates at Various Stages

Regeneration Stage	Mean Generation Time (hr)[a]
I	204, 223, 431
II	147, 168, 169, 186, 192, 195, 260
III	131
V	107
VII	72, 90
VIII	65
IX	67, 100
X	49, 55
XI	50, 52, 57, 80

[a]Each value represents the mean cell generation time for each lens regenerate.

the proliferating zone indicates the presence of alpha and beta crystallins in stages later than IX but not earlier. It is quite possible that these synthetic activities of cells and their generation time are mutually dependent.

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[1]USPHS International Fellow.

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6.2 AUTORADIOGRAPHIC STUDIES OF DNA SYNTHESIS DURING THE DEVELOPMENT AND DIFFERENTIATION OF CHICK LENS

S. P. Modak
Georgia Morris[1]
Marion E. Roesel

Previous studies from this laboratory[2] on the regenerating lens in *Triturus viridescens* have suggested a negative correlation between DNA and crystallin synthesis. We are interested in knowing whether a similar relation holds in lens formation in other vertebrates. In this work the distribution pattern of DNA-synthesizing cells in developing chick lens was studied.

Material and Methods. – White Leghorn chick embryos were cultured in vitro by the double-ring culture technique[3] at various stages of chick lens development as described by O'Rahilly and Meyer.[4] Blastoderms were explanted on a medium composed of equal parts of Tyrode and thin albumen containing 2 μc/ml of ^{3}H-thymidine (specific activity 17.4 curies/millimole, New England Nuclear Corporation, Boston, Massachusetts). Embryos were incubated for periods of 1, 2, and 4 hr in the presence of label and then fixed in Carnoy's fixative (6:3:1). Sections were treated in cold TCA for 10 min. Slides were coated with liquid emulsion (NTB-3, Kodak), exposed in dark for seven days, and developed. Autoradiographs were counterstained with Meyer's hemalum and mounted in balsam.

In all, 80 successful experiments were carried out. We are reporting in this communication the analysis of 31 lenses. Nuclei from the presumptive or definitive lens epithelium, presumptive or definitive lens fiber area, and the adjacent head mesenchyme were counted to determine the percentage of labeling. Of these, the head mesenchyme served as the control. At least 1500 nuclei per lens were counted.

Results and Discussion. – Table 6.2.1 indicates the percentage labeling of nuclei in the various regions of lenses from stages 14 to 17.

It is obvious from the above data that the labeling frequency of the head mesenchyme and the lens epithelium are almost the same for any given duration of labeling. On the other hand, significant differences in the labeling frequencies exist between the presumptive lens epithelium and the presumptive lens fiber area. These differences at these early stages, well before definitive cellular differentiation, are of particular importance.

DNA synthetic activity in the lens was followed during the developmental sequence. Percentage ratios for labeling of nuclei in the external as well as the internal layer are plotted against the stage of development (Fig. 6.2.1). Fifteen lenses, treated for 4 hr with tritiated thymidine, were chosen for the comparison. The labeling frequency of the external layer remains fairly constant at least until

Table 6.2.1. Percentage Labeling of Nuclei – Stages 14 to 17

Duration of Labeling (hr)	Number of Lenses	Percentage of Labeling		
		Lens Fiber Area (%)	Lens Epithelium (%)	Head Mesenchyme (%)
1	9	16–23	30–45	30–45
2	6	23–38	50–60	50–60
4	8	28–38	50–70	57–71

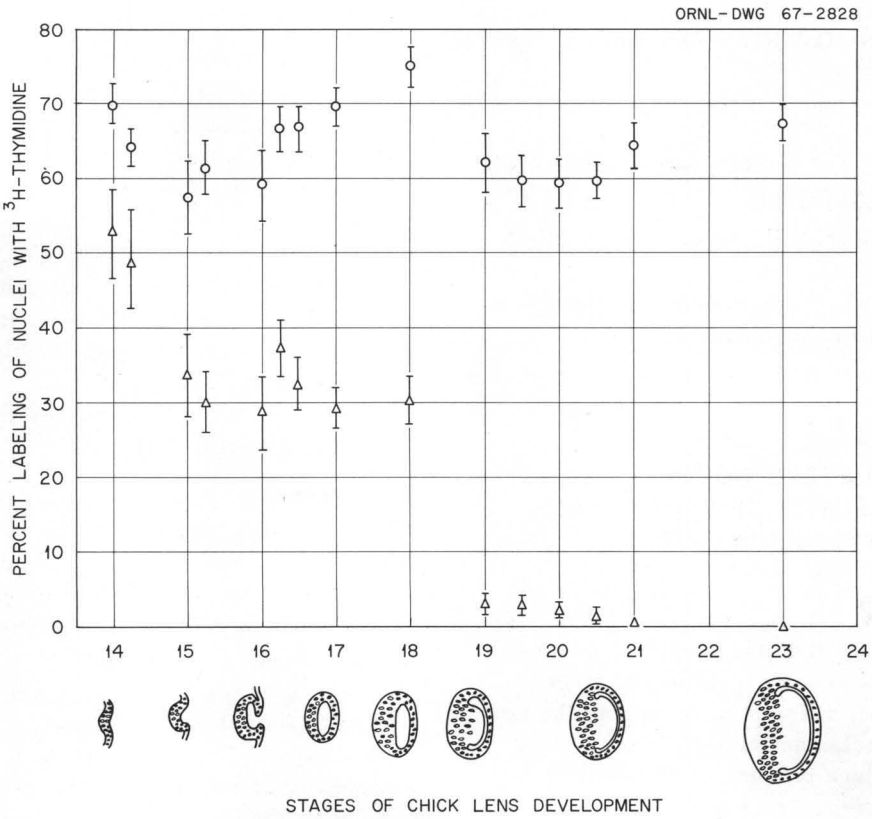

Fig. 6.2.1. DNA Synthesis During Lens Differentiation. —⊖—, presumptive/definitive lens epithelium; —Δ—, presumptive/definitive lens fiber area. Standard mean deviation at 95% confidence limit.

stage 23, while the labeling frequency in the internal layer steadily decreases up to stage 18, then sharply drops and reaches zero by stage 23. The most interesting feature revealed by this data is once again the decrease in the DNA synthetic activity already by stage 14 when no apparent cytodifferentiation is noticeable. Recently, Zwaan and Ikeda[5] have demonstrated the sequential appearance of various crystallins in the developing chick lens. Delta crystallins start appearing by stage 14 or 15 in the presumptive lens fiber area. We have demonstrated a small but appreciable decrease in the labeling frequency in this area compared with the presumptive lens epithelium area.

Zwaan and Ikeda have further shown that, by 3 to $3\frac{1}{2}$ days, alpha crystallins start appearing in the centrally located fiber cells and gradually spread in the surrounding cells. We find that it is around the same time that the lens fiber nuclei have completely stopped DNA synthesis. Thus our data suggest a decrease of labeling frequencies in the cells synthesizing delta crystallins, and cessation of DNA synthesis in the cells synthesizing alpha crystallins.

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6.3 IMMUNOELECTROPHORETIC ANALYSES OF THE PURITY OF *RANA PIPIENS* γ-CRYSTALLIN SAMPLES

D. S. McDevitt Isaura Meza

γ-Crystallin synthesis has been demonstrated by workers in this laboratory[1,2] to be the primary event of lens fiber differentiation in both the bovine mammal and urodelan amphibian. With the fractionation of the lens proteins of the anuran amphibian *Rana pipiens*,[3] an experimental organism with promising potential for developmental studies concerning the ontogeny and localization of γ crystallin in lens differentiation is now available.

It is proposed to demonstrate the presence of γ crystallin in the developing *R. pipiens* lens by means of the fluorescent antibody technique, using adult *R. pipiens* γ crystallin as antigen. Our primary concern at present is the preparation of a highly purified γ crystallin, so as to eliminate any future aspecific immunofluorescence when the antibody to the γ crystallin is collected, treated with the fluorescent reagent, and applied to sections of *R. pipiens* lenses at various embryonic stages.

Antisera to total lens protein were obtained by subcutaneous injection of a 1:1 total lens protein–Freund's adjuvant emulsion into virgin female rabbits. The antisera collected were treated with ammonium sulfate to produce a crude γ-globulin fraction, utilized as "antibody" in the immunoelectrophoresis procedures to follow.

The buffer systems used in immunoelectrophoretic investigations of the lens antigens obtained by chromatography were found to be of great importance in obtaining satisfactory resolution, that is, separation of the arcs. Several of the buffers cited in the literature were unsuitable for our purposes, providing little or no resolution, with varying ionic strengths and electrophoresis periods (e.g., Tris-borate-EDTA, pH 9.1; Veronal-HCl, pH 8.3). Tris-glycine (pH 8.4) and Veronal-acetate (pH 8.2) buffers were found to be satisfactory, but optimal resolution was obtained with Antoine's borate buffer, 0.14 *M*, pH 7.4. In addition, the electrophoretic medium used, agar, provided much better results than immunoelectrophoretic analyses in acrylamide (4.5% Cyanogum). Apparently the acrylamide presented an appreciable barrier to diffusion of the larger (in molecular weight) lens proteins, resulting in poor separation and uncharacteristic migration rates.

Preparation of pure γ crystallin as antigen, as judged by immunoelectrophoresis of the γ-crystallin sample vs anti-total-lens-protein antibody, has been obtained by column chromatographic methods. Ion exchange chromatography of total lens proteins, using DEAE-cellulose, resulted in the separation of a heterogeneous γ-crystallin fraction, as exhibited by its behavior in disk electrophoresis; no α- or β-crystallins were detectable by this method. However, immunoelectrophoretic analyses of samples of the DEAE-cellulose γ-crystallin fraction vs anti-total-lens-protein antibody revealed a very faint second arc possessing the mobility characteristic of an α- or β-crystallin (Fig. 6.3.1*a,b*). In order to remove such an impurity, adult *R. pipiens* total lens protein samples were first fractionated by gel filtration on Sephadex G-100 into the three classical groups of lens proteins, the α-, β-, and γ-crystallins. The γ-crystallin fraction so isolated was then concentrated and rechromatographed on a DEAE-cellulose column. This γ-crystallin fraction was found to be free of the α-crystallin seen previously, producing only one arc vs anti-total-lens-protein antibody in

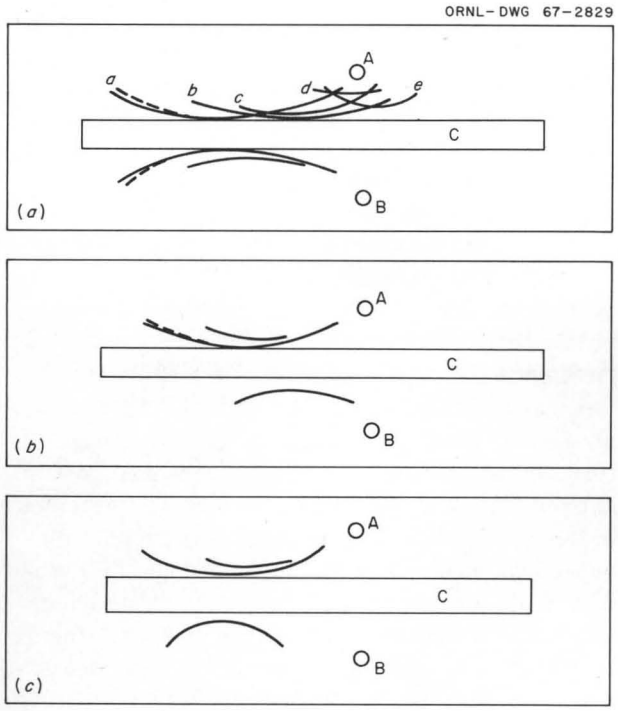

Fig. 6.3.1. Immunoelectrophoresis of *R. pipiens* Lens Antigens in 1% Agar (0.14 *M* Borate Buffer, pH 7.4, 320 v, 6–8 ma, 1 hr). (*a*) A – total lens protein; B – DEAE γ-crystallin fraction; C – anti-total-lens-protein antibody (*a*, γ-crystallin arc; *b*, *c*, *d*, and *e*, α- and β-crystallin arcs). (*b*) A – DEAE γ-crystallin fraction; B – DEAE α-crystallin fraction; C – anti-total-lens-protein antibody. (*c*) A – DEAE γ-crystallin fraction; B – γ-crystallin fraction obtained after Sephadex G-100 and DEAE-cellulose chromatography; C – anti-total-lens-protein antibody.

immunoelectrophoresis (Fig. 6.3.1c). Spur formation occasionally evident in the γ-crystallin arc indicates a heterogeneity of the γ-crystallin fraction, revealed in prior disk electrophoretic analyses. The difference in antigenicity, if any, of such suggested individual γ crystallins is as yet undetermined.

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6.4 RNA SYNTHESIS DURING LENS REGENERATION

Egidio Puccia D. H. Reese
Tuneo Yamada

Introduction. – Earlier autoradiographic studies[1,2] of RNA synthesis during Wolffian lens regeneration indicated that soon after lens removal enhancement in incorporation of ^{3}H-uridine and ^{3}H-cytidine into the ribonuclease-sensitive fraction of the nucleus of iris epithelial cells occurs. The enhanced level of incorporation is maintained during depigmentation and multiplication of regenerate cells. When the cells enter the fiber differentiation phase a gradual decrease of incorporation becomes evident. In the latter part of fiber differentiation phase the incorporation is not detectable. In the cells in multiplication or early fiber differentiation, the radioactivity is mostly localized in the nucleus within 3 hr after injection, but subsequently a considerable fraction of radioactivity becomes detectable in the cytoplasm. On the other hand, in the cells before depigmentation such spread of radioactivity into the cytoplasm cannot be detected, most of the radioactivity being restricted to the nucleus within 12 hr after injection of labeled precursors. Thus sequential changes in the pattern of RNA synthesis with the progress in cellular phases are suggested. In the present study RNA synthesis in the lens regenerate at various time intervals after lens removal was studied with the sucrose gradient technique.

Methods. – The lens regenerates were isolated from the eyes of adult *Triturus viridescens* 4, 10, and 20 days after bilateral lentectomy, In the 4-day and 10-day series the marginal dorsal iris was isolated, which includes all cells participating subsequently in lens formation. In the 4-day series all of those cells are still heavily pigmented, while in the 10-day series a part of those cells have been depigmented. In the 20-day series the majority of regenerates have formed small lenses with distinct epithelial and fiber areas. In this series the iris contained both iris epithelium and stroma. The isolation of regenerates from the eye was conducted with microscissors and microforceps under a binocular microscope. For one sucrose density gradient experiment about 120 regenerates were used.

The regenerates were cultured in a slightly modified Wolff-Quimby amphibian culture medium to which 2 μc/ml of ^{14}C (U)-uridine was added.

Culture was conducted under constant stirring for 30 min, 2 hr, and 5 hr, at 28°C in 5% CO_2 in air. The regenerates were washed and extracted with a mixture of solution of sodium naphthalene disulfonate containing sodium polyvinyl sulfate and 90% phenol containing quinoline at 4°C. The regenerate RNA fraction thus obtained was mixed with carrier RNA, which was isolated either from the brine shrimp eggs or from the frog liver with a modified method of Ralph and Bellamy.[3] The mixture was precipitated and washed in ethanol and acetone, and later studied on a linear gradient from 5% to 20% sucrose layered on top of 60% sucrose, in the presence of sodium polyvinyl sulfate.

Results and Discussion. – Some of the sucrose density gradient patterns obtained are reproduced in Fig. 6.4.1. Comparing the radioactivity data of 4-day and 21-day series, the presence of 28 S and 18 S peaks in the 21-day series and their absence in the 4-day series are obvious and seem to suggest that synthesis of ribosomal RNA starts in the intervening stage. This is in conformity with the autoradiographic data cited in the introduction as well as with the electron microscopic observation demonstrating an increase in ribosomes during the multiplication phase.[3,4] In all cases of the 4-day series a prominent peak at 4 S was consistently observed. Whether this indicates preferential de novo synthesis of 4 S RNA, or terminal addition to the preexisting 4 S RNA cannot be decided at the present stage of investigation. In the 10-day series a prominent peak localized between 18 S and 4 S regions was observed. In the control with ribonuclease, radioactivity disappeared completely in the tubes younger than No. 23 and did not indicate any peak. Thus observed radioactivity curves should be due to RNA. The optical density curve of the control series showed a small peak at around 16 S, whenever brine shrimp RNA was used as a carrier. It is known that this RNA sample is contaminated with DNA. When the frog liver RNA was used as a carrier, the optical density curve of ribonuclease-treated material ran parallel to the radioactivity curve without indicating any peak.

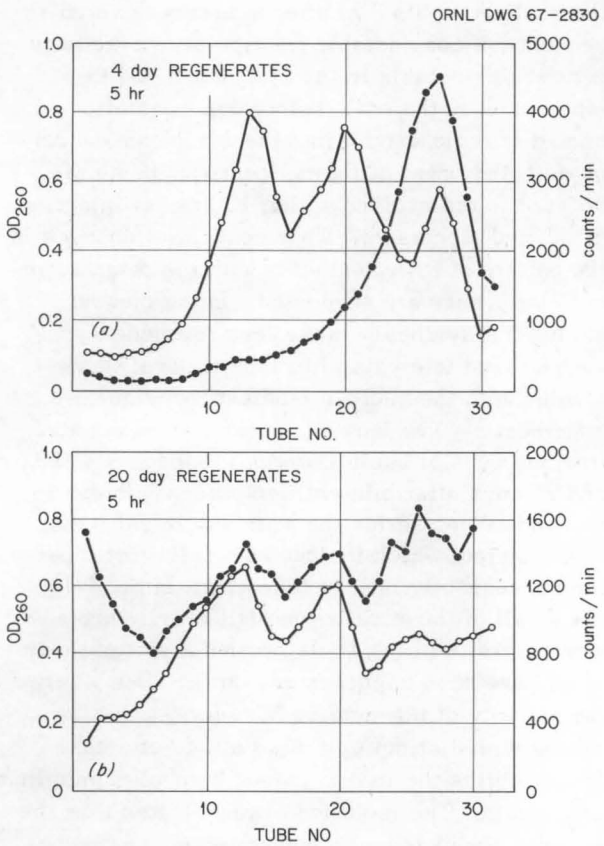

Fig. 6.4.1. Sucrose Density Gradient Patterns. (*a*), labeled RNA of 4-day lens regenerate mixed with frog-liver RNA as a carrier. –○–○–, optical density; –●–●–, radioactivity. (*b*), labeled RNA of 21-day lens regenerate mixed with brine-shrimp RNA as a carrier.

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6.5 THE MEMBRANOUS COMPONENT OF *TRITURUS* OOCYTE NUCLEOLI

O. L. Miller, Jr.

A membranous component attached to the DNA-containing core of peripheral nucleoli in oocytes of the spotted newt, *Triturus viridescens*, was reported previously.[1] This structure has been studied further using negative staining for electron microscopy. Preparative procedures consisted in

separating the core and cortex portions of nucleoli in a 0.025 *M* sucrose solution, centrifuging the cores onto an E.M. grid, brief fixing with formalin, and negatively staining with a thin film of 4% potassium phosphotungstate.

The membranous component characteristically is present either as an elongated tubule or a nearly spherical vesicle. In some cases, the component is divided into two or three somewhat smaller intact vesicles or appears as a string of several much smaller vesicles. In tubular form the component varies from 2.5 to 9 μ in length and 0.1 to 0.4 μ in width with the longer elements being the more narrow. The vesicles range from 0.3 to 1.3 μ in diameter and usually contain scattered granules near 100 A in diameter. The vesicles also often contain one or two opaque bodies varying from 0.1 to 0.2 μ in diameter and one to several short lengths of membrane. These inner membranes, as well as the limiting membranes of both the tubules and vesicles, appear double in favorable views and measure 70 to 100 A in thickness.

As noted earlier,[1] the location of the membranous component in these nucleoli is roughly coincident with the site of the initial incorporation of ^{3}H-uridine into ribosomal precursor RNA. Autoradiographic studies are in progress to determine the role, if any, of this component in the RNA metabolism of these nucleoli.

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6.6 BULK ISOLATION OF AMPHIBIAN OOCYTE NUCLEI

O. L. Miller, Jr. George Gassner III

The amphibian oocyte nucleus contains several hundred peripheral nucleoli which are not attached to chromosomes. These nucleoli contain DNA and function in the massive ribosomal RNA synthesis typical of these cells. Thus, this cell provides a unique opportunity to collect the DNA, transcriptive product, and associated enzymes of a single genetic locus. Because of the large size of these cells and their nuclei, however, established methods of cell fractionation generally have given yields of nuclei too low to allow separation and analysis of nuclear components. This report describes a technique which has given reasonable yields of clean nuclei in pilot runs.

The ovaries from an animal first are manually shredded with tweezers into small clusters of eggs and funneled into a thick-walled Teflon tubing with an inside diameter slightly smaller than the diameter of the oocytes. The eggs then are propelled rapidly past a nicking pin at the end of the tubing. The eggs are caught in a collecting chamber and pressed on a stainless steel screen having holes small enough to retain the oocytes but large enough to pass the expressed nuclei. A lower screen with smaller holes allows separation of nuclei and yolk platelets. Follicle cells and connective tissue remain with the pressed eggs. Using available screening which was not of optimum mesh size, runs with *Rana pipiens* eggs have given yields of 25%. Refinement of the process should allow recovery of 50% or more of the oocyte nuclei.

6.7 ULTRASTRUCTURAL STUDIES ON THE CENTRIOLE ADJUNCT IN DEVELOPING SPERM OF THE WASP *MORMONIELLA VITRIPENNIS* (WALKER)[1]

George Gassner III

Introduction. – During spermateliosis in the haploid and in the diploid forms of the wasp *M. vitripennis* the axial filament complex centriole, located at the base of the nucleus, is embedded in a chemically undefined structure of unknown origin and function. This structure is called the centriole adjunct and is a common feature of many insect sperm. This is the first time such a structure has been reported in a hymenopteran sperm (Fig. 6.7.1).

Gatenby[2] suggested that the centriole adjunct arises from the centriole matrix, whereas Barker[3] has speculated that the centriole adjunct consists at least partially of RNA and has a direct nuclear origin.

Discussion. – Light-microscope phase contrast observations of *M. vitripennis* sperm in 0.1 *M* phosphate buffer at pH 7.2 and bright field observations of formalin-fixed, methyl green–pyronin-stained smears were made. These approaches did not reveal any structure at the base of the sperm

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Fig. 6.7.1. A Longitudinal Section Through the Posterior End of a Late Haploid Spermatid Nucleus (N) Showing the Axial Filament Complex Centriole (C) and the Centriolar Adjunct (A).

head which could be distinguished from the base of the nucleus and the anterior part of the tail. Electron micrographs were made of sperm exposed to RNase digestion in order to test for the occurrence of RNA in the centriole adjunct. Prior to RNase digestion the testes were fixed in either 10% formalin or 2% glutaraldehyde. After the digestion period untreated testes and RNase-treated testes were washed in cold 5% TCA. Micrographs of both RNase-treated and untreated centriole adjuncts appeared partially extracted when compared with micrographs of centriole adjuncts which had been prepared by routine glutaraldehyde–osmium tetroxide fixation. Since both the treated and untreated specimens appeared extracted, an interpretation regarding possible RNA content is not warranted at this time.

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6.8 CRUSTACEAN LIPOVITELLIN: ISOLATION AND CHARACTERIZATION OF THE MAJOR HIGH-DENSITY LIPOPROTEIN FROM THE EGGS OF DECAPODS

R. A. Wallace Susan L. Walker

The eggs of many of the crustaceans have long been known for their intense and distinctive coloration, ranging all the way from purple to red. When preserved or "fixed" for cytological study, those eggs which display colors associated with the shorter wavelengths of the visible spectrum undergo a color change to orange or red. We have now been able to demonstrate that the cellular agent primarily responsible for this variable pigmentation is a carotenoprotein. By extracting crustacean eggs or mature ovaries with 0.5 *M* NaCl and subsequently recovering from the extract that material which precipitates between a 45 and 65% ammonium sulfate concentration, we have obtained at least 70% of the protein originally present as a relatively homogeneous lipoprotein as judged by salt fractionation data, gel filtration, and ultracentrifugation.

Absorption spectra recorded for 0.1% solutions of representative lipoproteins document a hyperchromatic effect in the visible region when the solvent is made alkaline (Fig. 6.8.1). In addition, the green protein of *Homarus* and the purple protein of *Uca* undergo spectral shifts resulting in an orange solution, whereas the orange protein of *Cancer* retains its original coloration. The coloration in the visible region is entirely due to a carotenoid noncovalently associated with the rest of the lipoprotein, and, presumably, the carotenoid present in *Cancer* is either of a different type, or else is already in a state which is achieved in the other forms only after a configurational change in the protein.

Sedimentation-velocity runs in the ultracentrifuge on proteins isolated from the eggs of six different crustaceans (Fig. 6.8.2) suggested a similar concentration dependence [$d\,(100/S_{20,w})/dc = 6.32 \times 10^{-2}$ cm^3 S^{-1} mg^{-1}]. Corrected sedimentation values, diffusion coefficients calculated from K_D values on a 6% agarose column according to the method of Ackers,[1] and partial specific volume data determined pycnometrically were used to calculate molecular weights for the six proteins (Table 6.8.1). The average molecular weight was found to be 3.5×10^5, and an average frictional ratio (f/f_0) was also calculated as 1.1, suggesting a fairly symmetric shape for all the proteins.

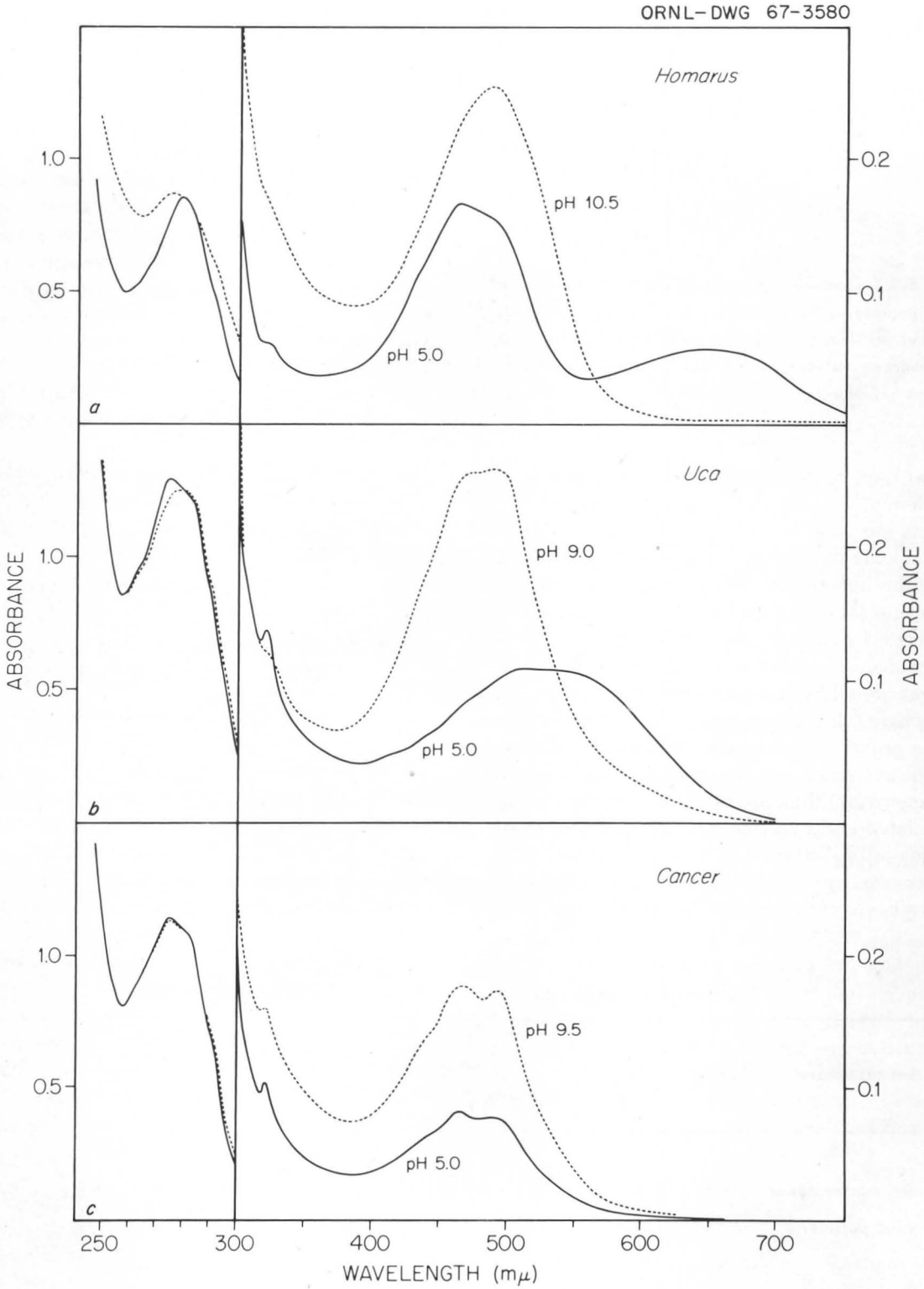

Fig. 6.8.1. Ultraviolet and Visible Absorption Spectra for *Homarus* (*a*), *Uca* (*b*), and *Cancer* (*c*) Egg Lipoproteins in 0.5 *M* NaCl – 5 mM EDTA at pH 5 (Solid Line) and pH 9–10.5 (Dashed Line).

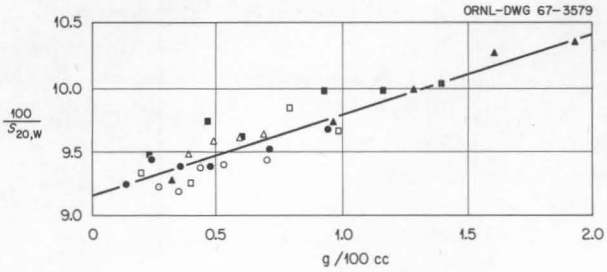

Fig. 6.8.2. Sedimentation Coefficient of Crustacean Egg Lipoproteins as a Function of Concentration: (○) *Pagurus*; (●) *Uca*; (△) *Sesarma*; (▲) *Cancer*; (□) *Libinia*; (■) *Homarus*. Solvent: 0.5 *M* NaCl – 5 mM EDTA (pH 5). The line represents the least-squares fit to all the points.

Total lipid content, protein nitrogen, protein phosphorus, and absorptivity values for the six proteins are also provided in Table 6.8.1. These data indicate an average lipid content of 30% and, unlike the lipoproteins found in vertebrate eggs, a complete lack of protein-bound phosphorus.

A single lipoprotein from the mature ovaries and early embryo of six different decapods has thus been recovered and characterized in each case as the primary (although perhaps not exclusive) yolk protein of these crustaceans, and we have designated it as crustacean lipovitellin. Vitellogenesis, and presumably the deposition of lipovitellin, can be initiated in dormant females by either eyestalk ablation or implantation of thoracic ganglia.[2] Furthermore, cytologic evidence[3] indicates that the synthesis of yolk protein in crustaceans occurs within the oocyte itself (unlike the case for vertebrates).

It would appear, therefore, that the vitellogenic ovary of the crustacean represents a tissue which is synthesizing primarily a single lipoprotein, and this synthetic mechanism can in turn be brought under experimental control. Future work will thus be directed toward the study of the crustacean lipovitellins as models of lipoproteins in general, both in terms of their interesting structural and chemical characteristics, and in reference to the manner of their synthesis, assembly, and transport within the cell.

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6.9 VITELLOGENESIS IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

J. N. Dumont

Recent studies of oocyte development in several animal forms indicate that a variety of cellular processes are involved in vitellogenesis. In some organisms the appearance of deutoplasmic units within the ooplasm is attributed to synthesis by an organelle system or systems,[1] whereas in others

Table 6.8.1. Physical and Chemical Data for Crustacean Egg Proteins

Animal Source	$S^0_{20,w}$ (S)	$D^0_{20,w}$ (cm^2/sec)	$\bar{v}$ (cc/g)	Molecular Weight	Lipid (%)	Protein Nitrogen (%)	Protein Phosphorus (%)	Absorptivity at 280 mμ (liters g^{-1} cm^{-1})
		$\times 10^{-7}$		$\times 10^5$				
Homarus americanus	10.8	4.1	0.82_0	3.6	29	15.3	<0.01	0.85
Pagurus pollicaris	11.1	3.9	0.81_5	3.7	33	13.6	<0.01	1.18
Uca pugilator	10.9	4.4	0.82_3	3.4	32	14.6	<0.01	1.25
Sesarma reticulatum	10.8	4.0	0.81_4	3.5	29	15.0	<0.01	1.39
Cancer irroratus	10.9	4.3	0.81_5	3.3	28	14.8	<0.01	1.11
Libinia emarginata	11.0	4.0	0.81_9	3.7	30	13.8	<0.01	0.76

it has been established that they arise by the micropinocytotic uptake of extraoocytic components.[2,3] In still other forms vitellogenesis involves a combination of these two mechanisms.[4] In an effort to compare the vitellogenic processes in various animal groups and to determine, if possible, any basic features common to all, the process of oocyte maturation, particularly with respect to vitellogenesis, is being investigated in the horseshoe crab, *Limulus polyphemus*, phylogenetically an ancient arthropod.

The oocytes of Limulus are suspended from the walls of the ovarian tubules by a slender cellular stalk. The ovarian tubule, stalk, and oocyte are covered externally by a layer of filamentous material referred to as the tunica propria. There are no follicle cells. The oocytes are divided into two general categories: (1) previtellogenic and (2) vitellogenic. The ooplasm of previtellogenic oocytes is characterized by ribosomes, large glycogen rosettes, small mitochondria, elements of rough and smooth endoplasmic reticulum, and a large centrally located nucleus. The most conspicuous organelle system is the Golgi complex, which at this stage is located in a circumnuclear position. The surface of the previtellogenic oocyte is adorned with short microvilli. There is little evidence of micropinocytotic activity in these cells.

During the vitellogenic period, the beginning of which is marked by the appearance of the vitelline envelope, marked changes occur in the oocyte. At the surface, the microvilli elongate and branch. Micropinocytotic processes are initiated but are not pronounced until later stages. The Golgi complexes enlarge and migrate peripherally to a position just beneath a rim of cortical cytoplasm which remains essentially devoid of organelles throughout vitellogenesis (Fig. 6.9.1). A close relationship is established between elements of smooth endoplasmic reticulum and membranes of the Golgi complex. This relationship suggests the transfer of material, via coated vesicles, from the former to the latter (Fig. 6.9.2*a*). Yolk bodies begin to form in these Golgi regions and at first appear as a collection of small coated and uncoated vesicles surrounded by a membrane (Fig. 6.9.2*a*). As they mature, they acquire a core of dense material. Finally, as mature yolk bodies they are dense, rather homogeneous structures (Fig. 6.9.2*b*).

During the final stages of vitellogenesis, micropinocytosis becomes pronounced and the Golgi complex diminishes in size. At this stage the direct fusion of internalized micropinocytotic vesicles gives rise to yolk bodies which are morphologically indistinguishable from those formed by the oocyte organelles. Thus, it appears that the initial accumulation of yolk occurs by intraoocytic processes through the collected efforts of the endoplasmic reticulum and the Golgi complex. Later, during the final stages of vitellogenesis, yolk or its precursors are internalized from an extraoocytic source(s) by micropinocytosis. Vitellogenesis in this form is therefore a two-step process which involves the "packaging" of yolk components presumably synthesized by the oocyte as well as the accumulation of components from other sources.

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6.10 COMPARATIVE CHROMATOGRAPHY OF THE YOLK PROTEINS OF TELEOSTS

D. W. Jared R. A. Wallace

In a continuing study of the yolk proteins of vertebrates, we have used TEAE-cellulose chromatography as a method of purifying and characterizing the yolk proteins of avians, reptiles, amphibians, and fishes. The eggs of all such oviparous vertebrates, with the exception of the teleosts, have either granular or platelet yolk proteins which lend themselves well to the chromatographic system we have developed.[1] The eggs of teleosts, however, contain a fluid yolk, the protein components of which do not adhere to the adsorbent when the standard buffer system (10 *mM* citric acid–60 *mM* 2-amino-2-methyl-1-propanol) is used. However, a decrease in the concentration of the starting buffer by a factor of 4 proved to be a successful modification of the original method when applied to teleosts:[2] of the 17 species we

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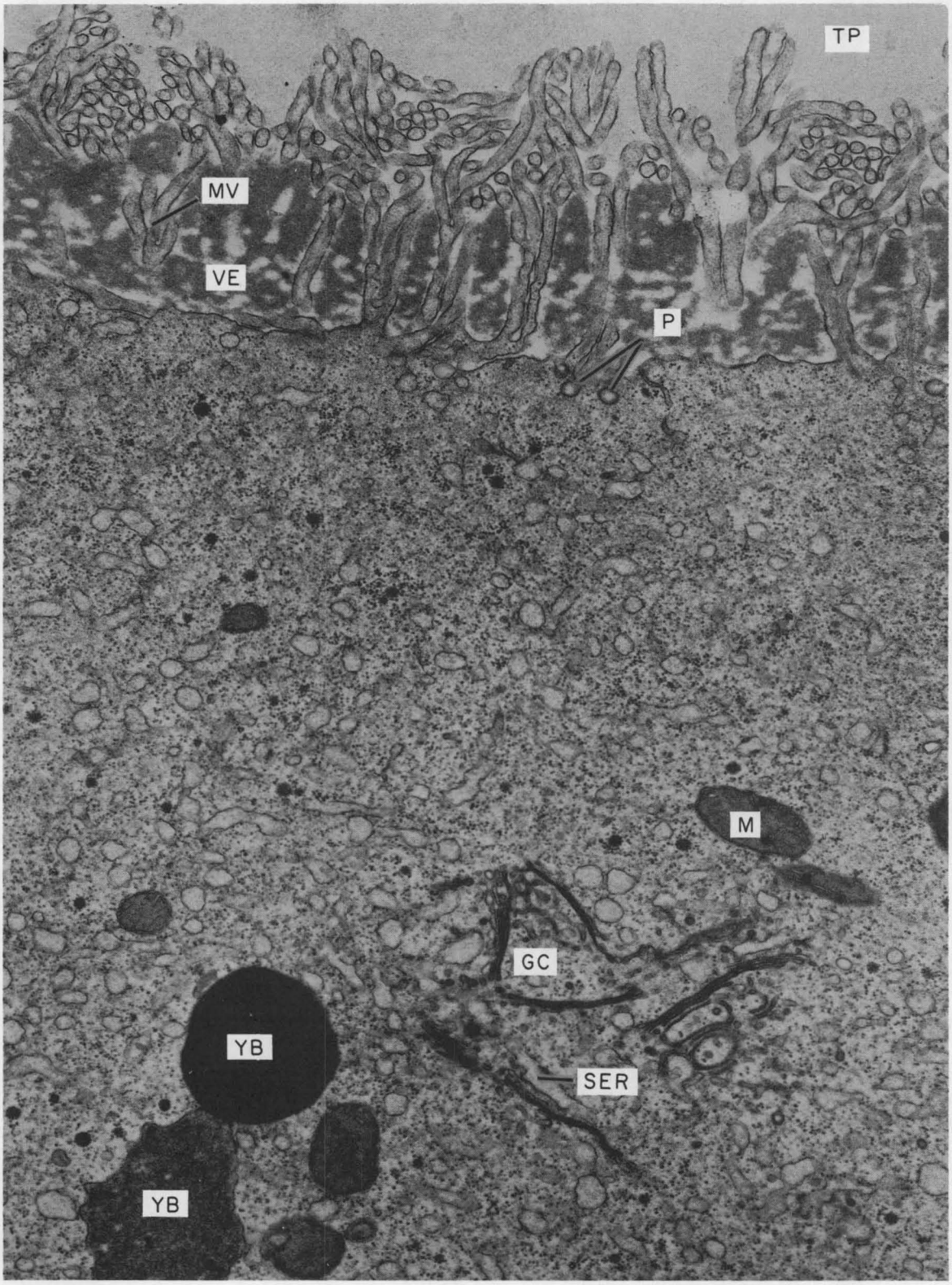

Fig. 6.9.1. An Electron Micrograph Showing the Surface and a Portion of the Cortical Ooplasm of an Oocyte in the Early Vitellogenic Stage. The egg is covered externally by the tunica propria (TP). Microvilli (MV), many of which branch, pass through channels in the dense vitelline envelope (VE). Micropinocytotic pits and caveolae may be seen at the surface (P). A Golgi complex (GC), elements of the smooth endoplasmic reticulum (SER), yolk bodies (YB), and a mitochondrion (M) are depicted lying beneath the rim of cortical cytoplasm.

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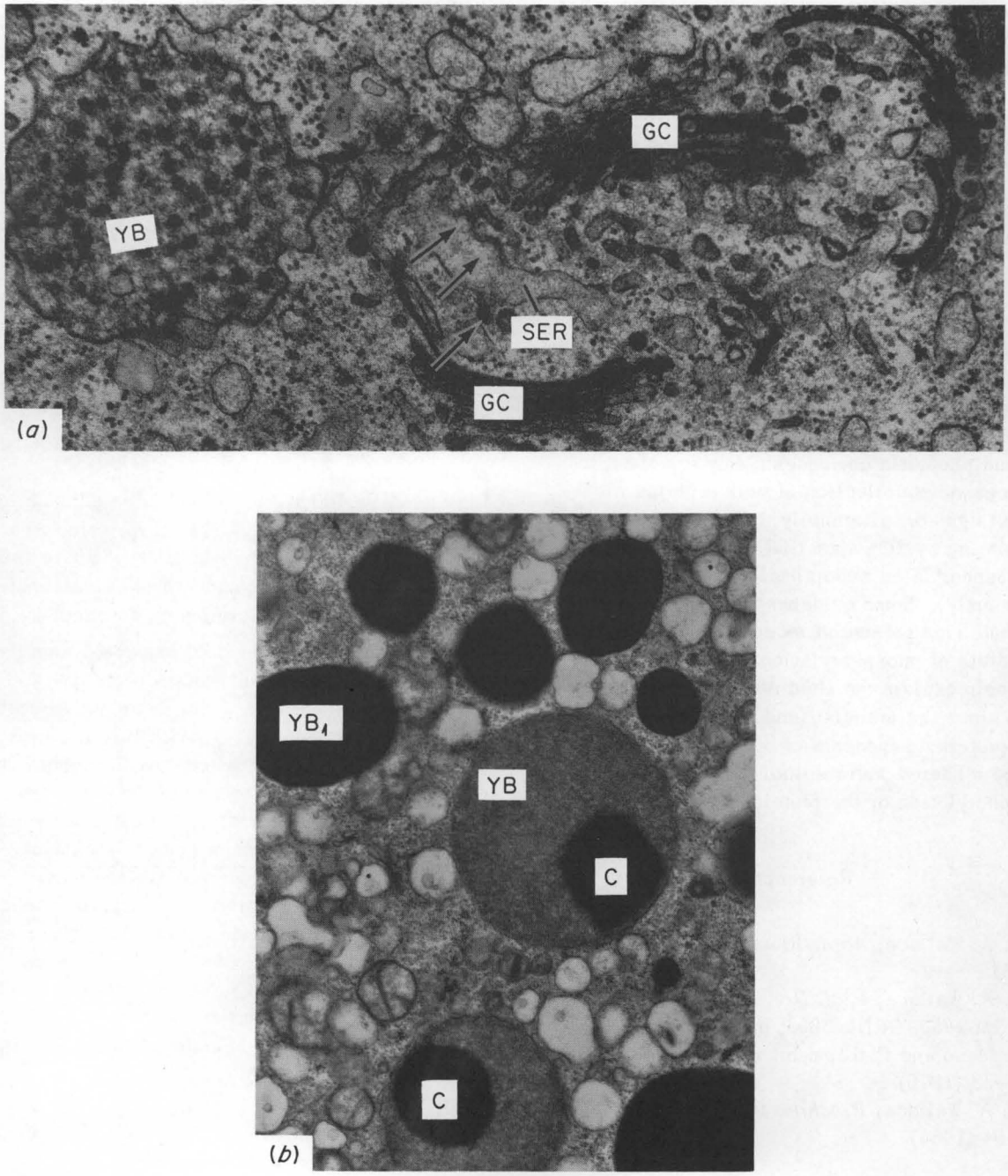

Fig. 6.9.2. (*a*) The Relationship Between the Golgi Complex (GC) and Smooth Endoplasmic Reticulum (SER). Blebs on the membrane of the endoplasmic reticulum coated with a dense material are indicated at the arrows. These may be involved in the transport of material from the endoplasmic reticulum to the Golgi complex. A developing yolk body, composed of coated and uncoated vesicles surrounded by a membrane, is labeled YB.

(*b*) Steps in the Maturation of a Yolk Body Such as That Shown in *a*. The small vesicles gradually disappear and a core of dense material (C) develops within the body (YB). The mature yolk body is somewhat smaller and electron dense (YB_1).

have now examined, the yolk proteins of all but three (mackerel, menhaden, and flounder) readily adhered to the TEAE-cellulose column and could be subsequently eluted with the usual gradient system. From the yolk of those forms which could be successfully chromatographed (alewife, trout, mud minnow, stickleback, pipefish, killifish, golden shiner, sheepshead minnow, silversides, crappie, sauger, butterfish, and toadfish), one to multiple lipoprotein components together with one or several phosvitin components were recovered. The lipoproteins, upon chemical analysis, proved to be less phosphorylated than those isolated from the eggs of other vertebrates, and the extent of phosphorylation was roughly proportional to the elution position of each component upon chromatography.

The existence of multiple lipoprotein (lipovitellin) and phosvitin components may represent a heterogeneous collection of yolk proteins in teleost eggs or, alternately, a defective phosphorylating system such that a homogeneous protein is phosphorylated suboptimally (and hence heterogeneously). Some evidence for the latter postulate has been presented recently.[3] The relatively poor state of phosphorylation in teleosts would also help explain the fluid nature of their egg yolk, since the granular (and relatively insoluble) yolk-protein components of other vertebrate forms can be rendered water-soluble by a partial dephosphorylation of the proteins.[4]

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6.11 A LIPOPROTEIN FROM HEMOLYMPH AND OOCYTES OF THE BLUE CRAB

Marilyn S. Kerr

The site of yolk protein synthesis in the Crustacea is unknown. Beams and Kessel[1] concluded from their electron microscopic observations of crayfish oocytes that proteinaceous yolk granules are synthesized in the stacked cisternae of the endoplasmic reticulum. However, several investigators[2] studying the hemolymph of various crustaceans suggested that the egg yolk proteins of these animals are synthesized external to the oocyte. So far, there have been no definitive studies of specific hemolymph proteins similar to egg yolk proteins in these invertebrates.

Previous work[3] with the blue crab, *Callinectes sapidus,* indicated the presence of a yellow protein in hemolymph taken from some adult females. Separation of this carotenoprotein from other hemolymph proteins was accomplished by continuous-flow paper curtain electrophoresis. The major soluble lipoprotein of homogenized blue crab ovaries or oocytes was isolated by a combination of ammonium sulfate precipitation and ultracentrifugation (105,400 $\times$ *g* for 20 hr) in a sodium bromide solution (density = 1.4). The lipoproteins from the two sources were completely cross reactive in immunodiffusion analyses and had identical mobilities in starch gel and acrylamide electrophoresis.

The lipoprotein can also be extracted from the oocytes by following the general procedure developed by Wallace *et al.*[4] for isolating the major lipoprotein component or lipovitellin from eggs of six different decapod crustaceans. Preliminary results of physical and chemical determinations indicate that the blue crab lipovitellin is similar to those lipovitellins investigated by Wallace *et al.*[4] In conjunction with continuing these studies, ^{14}C-labeled amino acids will be administered to blue crabs undergoing vitellogenesis in an attempt to determine whether the lipoprotein found in the hemolymph is being transferred to the ovary after synthesis in the hepatopancreas or is resorbed by the hemolymph after synthesis within the oocyte.

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6.12 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.

Results and Discussion. – By comparing the MAK column patterns from epithelial cells 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 cells 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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6.13 THE STABILIZATION OF mRNA IN LENS EPITHELIAL CELLS

J. A. Stewart John Papaconstantinou

Studies on the regulation of protein and nucleic acid synthesis in lens cells have shown that, upon the differentiation of the epithelial cell to the fiber cell, mRNA becomes stabilized and protein synthesis is not affected by actinomycin D.[1] The maturation of epithelial cells to fiber cells in the lens represents the terminal stage of differentiation in this tissue. One of the characteristics of the fiber cell is that it has lost its ability to replicate. This cell, therefore, is in a permanent stationary phase. One of the questions that we have recently studied is whether the stabilization of mRNA occurs in cells which have not lost their replicative ability but are in a stationary phase. Such a cell may be found in the epithelial layer of the adult lens.[2,3]

Studies on the mitotic activity of epithelial cells in embryonic, early postnatal, and adult lenses have shown that the cells of the embryonic lens have a high mitotic index and, as the lens reaches its maximal size in the adult, the mitotic index is essentially negligible.[2,3] These cells, in the adult, are stationary and will remain in an extended G_1 phase of the cell cycle indefinitely. This stationary, extended G_1 phase is reversible, however, and cells in this phase can be induced to divide again by either physical damage or by placing them in tissue culture.

In the course of our studies on the biochemical properties of epithelial cells and fiber cells, it became apparent that the epithelial cells of the adult lens have properties more similar to fiber cells than to epithelial cells of young lenses or actively dividing cells in culture.[4] In view of this, experiments were carried out to determine whether protein synthesis by cells in the stationary, extended G_1 phase occurs on stable templates and whether the degree of stability in these cells is greater than that of the actively dividing cells in young lenses.

Results and Discussion. – Lenses from bovine embryos, newborn calves, and adult cows were used in these experiments. In each case the rate of incorporation of radioactive amino acids into proteins and the rate of incorporation of radioactive uridine into RNA was measured in the presence and absence of actinomycin D (30 μg/ml). The epithelial cells from control and actinomycin-D-treated lenses were homogenized, and the α-, β-, and γ-crystallins were fractionated on DEAE-cellulose columns. The α-, β-, and γ-crystallin fractions eluted from each column were counted to determine the degree of inhibition of protein synthesis by the actinomycin D. A summary of these experiments is shown in Table 6.13.1. It can be seen from these data that protein synthesis in the embryonic cells is strongly inhibited by the antibiotic and that the synthesis of these same proteins in the adult epithelial cells is essentially unaffected. The data also show that there is a progressive decrease in the ability of actinomycin D to affect protein synthesis as the lenses age.

Experiments were also done to determine the degree of inhibition of RNA synthesis in the presence of actinomycin D in embryonic, newborn, and adult epithelial cells. Phenol-extracted RNA was layered over sucrose gradients, and after centrifugation the fractions collected from the gradient were counted. It was found that in all cases the actinomycin D virtually stopped all RNA synthesis. These data indicate that as a result of the inhibition of RNA synthesis in the embryonic epithelial cells protein synthesis was also inhibited. Under similar conditions in which RNA synthesis in the adult epithelial cells was inhibited, protein synthesis was not affected. The results of these studies indicate therefore that epithelial cells in the stationary phase employ a mechanism in which the RNA templates are stabilized.

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Table 6.13.1. The Effect of Actinomycin D on Crystallin Synthesis in Bovine Lens Epithelial Cells

Lens	Crystallin Fraction	Radioactivity (dis/min per milligram of protein)		Percent Inhibition (–) or Stimulation (+)
		Control	Actinomycin (30 μg/ml)	
Embryo	α	46,262	15,590	–65
	β	22,326	10,168	–54
	γ	17,060	9,884	–42
Calf	α	3,796	1,948	–49
	β	2,046	1,123	–45
	γ	3,896	2,340	–40
Adult	α	4,827	4,508	–7
	β	5,136	5,136	0
	γ	8,172	8,172	–7

6.14 IMMUNOFLUORESCENT LOCALIZATION OF α-, β-, AND γ-CRYSTALLINS IN BOVINE LENS CELLS DURING GROWTH AND DIFFERENTIATION

R. E. Shore John Papaconstantinou

Through biochemical studies, evidence has accumulated which indicates that the tissue-specific proteins of the lens are specifically localized in distinct regions of the lens. The synthesis of γ-crystallins, for example, is associated with fiber cell formation.[1,2] A survey of the lens proteins in fiber cells in embryonic bovine lenses has shown that the distribution of the α- and β-crystallins varies with the age of the animal.[3] Thus, throughout the growth and differentiation of the lens, the synthesis of individual crystallins is initiated and repressed at specific times. The initiation of γ-crystallin synthesis at the time of initiation of fibrogenesis is a clear example of the regulation of protein synthesis at a specific stage of cellular differentiation.

Since the population dynamics of the epithelial cells and fiber cells changes as the lens ages, that is, both mitosis and fiber cell formation are slowed down, experiments were carried out to determine whether there is, at these stages of growth and differentiation, a specific regulation of protein synthesis. This was done through the use of immunofluorescent techniques, which because of their sensitivity would show the exact distribution of the α-, β-, and γ-crystallins in the different regions of the lens. In these studies, therefore, attempts have been made to map out the distribution of the lens proteins in the regions of the epithelial cell layer and in fiber cells during fibrogenesis. These analyses were carried out with embryonic, neonatal, and adult lenses. By using lenses from various ages, we are able to study this tissue at a time when the epithelial cells are either actively dividing (embryonic) or in a stationary phase (adult). Similarly, at these various ages, the rate of formation of the fiber cell is very fast in the embryonic lens when compared with that seen in the adult lens.

Results and Discussion. – *The Distribution of Lens Crystallins as a Result of Growth and Differentiation.* – Experiments were carried out to show the distribution of the α-, β-, and γ-crystallins in epithelial and fiber cells at different ages. At any one age, the distribution of each group of crystallins was found to be unique. Table 6.14.1 summarizes the details of these observations. A general pattern of a progressive loss of all crystallins from the central region epithelial cells was observed. This loss begins sometime after 46 days and before 112 days of gestation. Thus, it can be concluded that crystallin synthesis in the epithelial cells occurs in early embryonic lenses but becomes localized to the cells of the peripheral region of the lens as the tissue ages.

With respect to the fiber cells, at all ages all three crystallins were found in these cells. These

Table 6.14.1. Distribution of Lens Crystallins[a]

Age	Crystallin	Region of Lens							
		Epithelium				Fiber			
		Central		Germinative		Elongation		Cortical	
		C	*N*	*C*	*N*	*C*	*N*	*C*	*N*
Fetal									
46 days	α	+	−	+	−	+	−	+	−
	β	+	+	+	+	++	++	++	++
	γ	+	−	+	−	+	−	++	+
112 days	α	+	−	+	−	++	−	++	−
	β	−	−	±	−	+	±	+	+
	γ[b]	·	·	·	·	·	·	·	·
Calf	α	−	−	±	−	++	−	++	−
	β	−	−	±	−	+	±	+	+
	γ	−	−	+	−	+	−	+	±
Adult	α	−	−	−	−	++	−	++	−
	β	−	−	++	−	++	±	+	+
	γ	−	−	±	−	+	−	+	±

[a](−) Protein is not detectable.
(+) Protein is detectable.
(++) Intensity of fluorescence is greatest.
(±) Transitional region for this protein.

C: Cytoplasm.
N: Nucleus.

[b]Data incomplete.

data indicate therefore that as the lenses age the synthesis of α-, β-, and γ-crystallins becomes localized in the germinative region, which is adjacent to the region where cellular elongation is initiated.

The Intracellular Localization of α-, β-, and γ-Crystallins in the Epithelial Cells and Fiber Cells. – Within the epithelial cells and fiber cells, the α-, β-, and γ-crystallins showed some indication of specific localization.

With respect to the α-crystallins, these proteins were specifically localized in the cytoplasm of both epithelial cells and fiber cells.

β-Crystallins were found in the nucleus and cytoplasm. In the epithelial cells these proteins were specifically localized in the cytoplasm. They are first detected in the nucleus in the cells of the region of elongation. This was found to be the case for all four ages studied. The β-crystallins are also seen in the nucleus of the fiber cells.

With respect to the intracellular localization of γ-crystallins, these proteins are also found in the cytoplasm and nucleus. They first appear in the cytoplasm of the cells of the germinative region. In the region of cellular elongation and in the region of cortex fiber cells, the γ-crystallins can be detected in the cell nucleus.

It can be seen from these observations that both β- and γ-crystallins are first detected in the cytoplasm and then appear in the cell nucleus. The β-crystallins appear in the nucleus at an earlier stage of fibrogenesis than do the γ-crystallins. Since this is the stage at which terminal cellular differentiation is initiated, it is possible that either the β- or γ-crystallins may be involved in the regulation of fibrogenesis. It is now quite obvious that the synthesis of all three groups of proteins is regulated such that this synthesis becomes localized, in older lenses, to a specific region of the lens. The mechanisms of these regulations and the significance of the progressive

localization of β- and γ-crystallins to the region of cells undergoing maturation are being studied.

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6.15 AN ANALYSIS OF THE PULSE-LABELED RNA IN BOVINE LENS EPITHELIAL CELLS AND FIBER CELLS

John Papaconstantinou Emilia M. Julku

Introduction. – In a continuation of studies on the stabilization of mRNA during fiber cell differentiation, experiments were carried out to determine the properties of rapidly turning over RNA in the epithelial and fiber cells. It was the purpose of these experiments to determine whether there are either qualitative or quantitative differences in the heavy and light species of pulse-labeled RNA in epithelial and fiber cells. In addition to this, it was also our purpose to determine whether there is a precursor-product relationship (in epithelial cells or fiber cells) between the heavy and light pulse-labeled RNA.

In these experiments lenses were pulse labeled with ^{14}C-guanidine or ^{14}C-uridine. The epithelial and fiber cells were separated at the end of the experiment, and each cell type was phenol-extracted. The phenol-extracted RNA was fractionated on sucrose density gradients, and each fraction was analyzed for radioactivity.

In experiments involving pulse-chase analyses, the radioactive precursor, either ^{14}C-guanidine or ^{14}C-uridine, was chased with 100 times the concentration of the radioactive precursor using nonradioactive material.

Results and Discussion. – *Sedimentation Properties of Pulse-Labeled RNA in Epithelial Cells and Fiber Cells.* – In each experiment the sedimentation properties of pulse-labeled RNA were analyzed on sucrose gradients. To get some indication of the sedimentation rates of light and heavy molecular weight species, 5 to 20% sucrose gradients were run for 24, 10, 7, and 5 hr at 24,000 rpm. In all cases, the 28s and 18s ribosomal RNA peaks were used as markers. Analysis of these centrifugations showed major radioactive peaks at 45s, 30s, 20s, and 7 to 14s regions. In no case could we find significant radioactivity coincident with the 28s and 18s ribosomal RNA peaks. There were also regions of radioactivity ranging from 80s to 50s.

A comparison of the pulse-labeled RNA in epithelial cells and fiber cells did not reveal any qualitative differences between the two cell types. The major significant difference was quantitative in that the rate of RNA turnover in the fiber cells was much less than that observed for the epithelial cells.

Because of the high degree of radioactivity in the region of 45s and 30s RNA, experiments were done to determine whether this RNA is ribosomal precursor. In these experiments lenses were pulse labeled for 3 hr and then chased for another 3 hr with the same nonradioactive precursor. Analyses were carried out on the pulsed and chased epithelial cells and fiber cells.

An analysis of the pulse-chase epithelial cells showed that only a negligible amount of the 45s and 30s RNA could be chased into the 28s and 18s ribosomal RNA peaks. In addition, it was found that a significant amount of RNA was chased into the 20s region, and the largest amount of chased RNA was found in the 7 to 14s region. Thus, there was a rapid decrease of all of the pulse-labeled RNA in the 80s to 30s regions and an increase in the radioactive RNA of the 20s and 7 to 14s region. These data indicate that there may be a precursor-product relationship between pulse-labeled RNA heavier than 28s and the lighter 20s and 7 to 14s RNA. Although there was an increase in the radioactivity of the 20s and 7 to 14s RNA, this increase did not account for the total amount of counts lost from the >28s RNA. Thus, a large part of the pulse-labeled RNA may be broken down to small, acid-insoluble fragments.

Similar analyses of the pulse-chase RNA from fiber cells were carried out. These experiments indicate that there is a significant difference in the chase pattern of the relatively small amount of rapidly labeled RNA in the fiber cells. It was found that none of the >28s RNA could be chased into the 28s or 18s ribosomal RNA. In this respect the epithelial and fiber cell chase patterns are similar. However, in the fiber cell no precursor-product relationship could be found. There was

no indication of an increase in the 20s or 7 to 14s RNA. An overall decrease in all regions of RNA was found. It was interesting to note, however, that the rate of decrease in radioactivity was greatest in the >28s RNA and slowest in the 7 to 14s RNA.

Taking this into consideration, it is possible that the pathway of degradation of rapidly turning over RNA in epithelial cells and fiber cells is qualitatively the same and that our ability to detect a precursor-product relation in the epithelial cells and not the fiber cells may be due to (*a*) the decrease in rate of heavy RNA synthesis as the stabilization of mRNA proceeds or (*b*) a decrease in the rate of breakdown of heavy RNA as a result of the stabilization of mRNA. Experiments are now in progress to study the mechanism responsible for this variation in the chase pattern of rapidly turning over RNA at two different stages of differentiation.

6.16 THE SYNTHESIS OF LENS PROTEINS BY EPITHELIAL CELLS IN TISSUE CULTURE

John Papaconstantinou J. W. Carr

Through the work of D. M. Schenck in this laboratory, we succeeded in culturing calf lens epithelial cells which, when treated with vitreous humor, would undergo a characteristic cellular elongation. The specificity of this reaction was indicated by the observation that RA cells (derived from human amnion) would not respond to treatment with vitreous. It was also found that the lens epithelial cells would not respond to aqueous humor, thus indicating that this response may be due to a specific component or components of the vitreous. Further studies have shown that upon treatment of the vitreous with collagenase and hyaluronidase before it is added to the culture medium will stimulate a stronger response, that is, more widespread cellular elongation.

Since we have now established conditions under which lens epithelial cells can be induced to either retain their epithelial cell characteristics, or to elongate, we have initiated a series of studies to determine whether the synthesis of α-, β-, and γ-crystallins, in these cultured cells, can be regulated. The experiments to be discussed here were designed to show whether primary cultures of lens epithelial cells can synthesize lens proteins when they are cultured under conditions in which the epithelial cells do not elongate. Our main purpose in carrying out these experiments is to study the regulation of tissue-specific protein synthesis.

Results. – *The Synthesis of Lens Proteins by Epithelial Cells in Culture.* – In these experiments, primary explants of calf lens epithelial cells (both germinative and central region cells) were placed in culture for two weeks. The cells were grown in Hanks's salts with Eagle's MEM and 30% calf serum to maintain the cells in their epithelial cell morphology. During the two-week culture period, the medium was changed regularly to eliminate any of the contaminating fiber cells. In addition, during this period of culturing there was a significant outgrowth of epithelial cells so that the size of the initial explant was negligible compared with the outgrowth which occurred in two weeks. At the end of the two-week culture period, the cells were washed well with Hanks's salts to eliminate the amino acid mixture and were incubated for 6 hr in a medium containing a mixture of ^{3}H-amino acids in Hanks's salts and 30% calf serum. After the 6-hr incubation period, the cells were again washed well with Hanks's salts 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 7.0 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. Thus, the ^{14}C counts could 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 this experiment 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 de-

termine any slight variation in elution properties, a gradient elution system was used in these experiments. The results of this experiment showed that: (*a*) the lens cells in tissue culture synthesize α-, β-, and γ-crystallins, (*b*) there was no difference between the ^{14}C and ^{3}H peaks, and (c) 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 γ-Crystallins by Cells in Tissue 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. Our preliminary data indicate, therefore, that the cells in culture can synthesize γ-crystallins similar to those found in the fiber cells.

Discussion. – It has now been established that primary epithelial cells in culture can synthesize α-, β-, and γ-crystallins. The synthesis of γ-crystallins has been analyzed in detail, and it appears that each of these proteins is synthesized to the same extent by cultured cells and fiber cells in vivo. We conclude from these experiments that after two weeks in culture the ability of lens cells to synthesize their tissue-specific proteins is still retained. Immunochemical analyses have indicated, however, that this is not the case for cells which have passed through several generations in culture. We propose to repeat these experiments with cells after each subculturing to determine (*a*) how long it takes for these cells to stop synthesizing crystallins and (*b*) the sequence in which this occurs.

The fact that γ-crystallins are synthesized in culture indicates that these cells may be taking on the biochemical properties of fiber cells. This may not be the case, however, since R. E. Shore has shown by immunofluorescent techniques that there are γ-crystallins in the equatorial epithelial cells (germinative region cells). Our observations on the growth of the primary explants indicate that it is the cells of the germinative and equatorial region which replicate when placed in culture. Since γ-crystallins are already present in these cells before they are placed in culture, the observations reported here may not indicate that the synthesis of γ-crystallins was induced in tissue culture or that the cells are taking on fiber cell characteristics. On the contrary, since no vitreous was added to this culture medium, cellular elongation was not observed. Experiments are now in progress to determine the pattern of crystallin synthesis in vitreous-treated cells.

6.17 STUDIES ON THE SUBUNIT COMPOSITION OF BOVINE LENS α-CRYSTALLINS

Winnie G. Palmer John Papaconstantinou

The α-crystallins of the bovine lens have been shown to have a molecular weight of 1×10^6 and to be composed of several species of low-molecular-weight ($\cong 2.5 \times 10^4$) subunits.[1,2] Dissociation and reassociation of these protein molecules have been achieved with 7 *M* urea, guanidine·HCl, and sodium dodecyl sulfate (SDS). The subunits can be isolated by fractionation on DEAE columns in the presence of 7 *M* urea.

The procedures used to isolate α-crystallins in highly purified forms involve isoelectric precipitation at pH 5 followed by fractionation on DEAE cellulose.[3] By the use of a stepwise elution system, the isoelectric precipitated α-crystallin can be resolved into five distinct fractions on DEAE cellulose. The molecular weights of these fractions vary from 0.7×10^6 to 1.2×10^6. No differences between the individual α-crystallins could be found by high-voltage electrophoresis of the peptides after proteolytic treatment. These observations indicated that these proteins may not have qualitative differences but does not rule out the possibility of quantitative differences with

respect to the number of subunit species found in each protein. Since the subunits can be fractionated on urea-equilibrated DEAE columns, the individual α-crystallins could be dissociated and fractionated into their constituent subunits so that any variation in the amount of individual subunits within the complex protein molecule may be detected. The results of these experiments are described below.

Results and Discussion. – Experiments were carried out to determine whether there are quantitative differences in the subunit content of the α-crystallins prepared by DEAE-cellulose fractionation. Each α-crystallin fraction was dissociated in 7 *M* urea, and the subunits were eluted from a urea-DEAE column. The elution system involved using an increasing concentration of NaCl and phosphate buffer in 7 *M* urea. Using this fractionation procedure, the subunits could be identified. Although the peaks eluted from the column are not homogeneous, there are clear-cut regions of elution which for the purposes of these experiments were designated as subunit regions I, II, and III. The variation in the amount of protein (subunits) found in these regions for the individual α-crystallins can be seen from the data in Table 6.17.1. Peaks E to I represent the intact α-crystallins prepared by isoelectric precipitation and fractionated on DEAE columns. It can be seen that the subunits of regions I and II are most concentrated in α-crystallin (peak E) and decrease progressively in the other α-crystallins, the lowest concentration of these subunits being found in peak I. The reverse is found with the subunit eluted in region III. This variation in subunit composition may explain the elution characteristics of the intact α-crystallins on DEAE-cellulose, which involves an increase in NaCl and phosphate buffer concentration. The α-crystallin (peak I) that requires the highest concentration of NaCl to be eluted from the column contains more of the subunit (III), which also requires the highest salt concentration to be eluted.

These preliminary data on α-crystallin subunits indicate that the heterogeneity of the α-crystallins is due to the quantitative differences in their subunit content. Studies on the synthesis of the individual subunits are being carried out in an attempt to learn more about the mechanism of regulation of synthesis of this complex protein.

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Table 6.17.1. The Subunit Composition of Adult Bovine Lens α-Crystallins

α-Crystallin Peaks from a DEAE Column	Subunit Peaks from a Urea Column (mg)			Total Protein (mg)	Percent of Each Subunit in		
	I	II	III		I	II	III
E	6.7	26.0	5.5	38.2	17.4	68.1	14.5
F	7.1	29.4	6.5	43.0	16.4	68.5	15.1
G	5.4	21.6	10.2	37.2	14.6	57.9	27.5
H	1.82	8.96	7.91	18.69	9.7	47.9	42.3
I	0.96	3.76	5.52	10.24	9.4	36.7	53.9

Radiation Microbiology and Microbial Genetics

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7.1 ACRIDINE EFFECTS IN IRRADIATED *NEUROSPORA CRASSA*

C. E. Terry

The effects of pre- and post-uv-irradiation treatment of *Neurospora crassa* conidia with acridine dyes are being studied. When conidia are pretreated with 1 μg/ml of proflavine, acriflavine, or acridine orange before 254 nm irradiation, the dyes all exert a protective effect. This protection is more pronounced at high uv doses (e.g., 10,000 ergs/mm^2). Protection is greatest with proflavine, while acriflavine and acridine orange provide less protection in that order. There does not appear to be a constant dose-reduction factor, since survival for treated samples drops initially at the same rate as that of untreated suspensions.

When uv-irradiated conidia are incubated at 37° in the dark, they lose photoreactivability in 120 to 150 min. In *Escherichia coli*, loss of photoreactivability has been correlated with a "dark" repair system which excises dimers.[1] Although photoreactivability is lost in Neurospora, repair is not evident, since no increase can be seen in survival of held samples over that of samples plated immediately following uv irradiation. This result could possibly be explained if conidia normally undergo dark repair after plating, since no differences in survival would then be detectable. Acridine dyes have been shown to interfere with host-cell reactivation and thymine-containing

dimer excision in *E. coli*,[2,3] two types of recovery involving dark repair. When dark incubation is carried out in 1 μg/ml of proflavine and samples are withdrawn and plated at intervals up to 4 hr, survival drops for the first 120 min and then remains constant at 180 and 240 min. The shape of the survival curve changes from one with a large shoulder to one with an essentially linear form. This suggests that proflavine prevents repair of damage which becomes irreparable as incubation time progresses.

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7.2 CORRELATION BETWEEN INCREASED RADIATION RESISTANCE AND INCREASED CELL SIZE OF THE BACTERIUM *ESCHERICHIA COLI*

D. E. Axelrod H. I. Adler

The bacterial mutant *Escherichia coli* HfrR5,[1] selected for increased resistance to the lethal effects of x rays, has been found to have a larger cell size than the radiation-sensitive parent, HfrH. In order to understand the mechanism of radiation resistance of this mutant, we have investigated the relationship between its increased resistance and increased cell size.

The mutant-to-parent ratios for some cell parameters are as follows:

DNA/cell	1.40 ± 0.14
RNA/cell	1.82 ± 0.31
Dry weight/cell	1.43 ± 0.23
Volume/cell	1.54 ± 0.17

The resistant mutant is, therefore, larger than its parent but is probably not diploid.

The mutated allele controlling resistance is linked to *argC* in conjugal mating but is not cotransducible with *metA* nor *malB*. This allele is, therefore, distinguishable from previously reported alleles controlling radiation resistance to x rays.

When the mutated allele is transferred by conjugation to radiation-sensitive small cells, the radiation-resistant recombinants are large cells. When the sensitive parent is grown under conditions that temporarily result in increased cell size, its radiation resistance also increases. Therefore, increased cell size resulting from genetic or physiological changes can be correlated with increased radiation resistance.

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7.3 A MUTATION AFFECTING THE SIZE, SHAPE, AND RADIATION RESISTANCE OF *ESCHERICHIA COLI* K-12

H. I. Adler Alice A. Hardigree

Escherichia coli K-12 P678 is a small rod-shaped bacterium. Samples of this organism from a log-phase culture consist of regularly shaped, rod-shaped cells undergoing division by constriction near the midpoint. We have treated this organism with the mutagen triethylene melamine and isolated several x-ray-resistant mutants from it. One of these, labeled P678-7, has an unusual morphology when observed during log phase by phase-microscope techniques. The cells of this mutant are extremely pleomorphic, ranging from plump rods to become amoeboid forms. Even the most unusual forms are capable of growth and division, and the culture as a whole grows only slightly slower than the nonmutant rod-shaped P678 parent. The unusual cell forms are not fragile. They survive dilution from complex media into distilled water. They form colonies on the surface of agar. They are lysed by lysozyme at only one-tenth the rate of the parental P678 culture. The distribution of cell forms is not affected by the incorporation of diaminopimelic acid or high concentrations of sucrose in the growth medium.

Cells of P678-7 retain the genetic markers and the ability to mate with donor strains, character-

istic of P678. Preliminary genetic analysis of recombinants indicates that the region controlling both radiation resistance and unusual morphology in this strain is not near the "lon" locus previously studied.[1] The available data suggest a locus(i) near those controlling threonine and leucine biosynthesis.

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7.4 A NEW CELL DIVISION MUTANT OF *ESCHERICHIA COLI*

H. I. Adler Alice A. Hardigree
Amikam Cohen D. P. Allison[1]

We have recently isolated a cell division mutant of *Escherichia coli*, K-12 P678, that may be useful in a variety of studies. In addition to undergoing normal cell division near the midpoint of the rod-shaped cell, cells of this organism frequently undergo a secondary division close to one pole (Fig. 7.4.1). This misplaced division results in the production of a class of very small cells. These small cells may be separated from the bulk of the population and analyzed. They have normal appearing walls and membranes and contain reasonable amounts of ribonucleic acid and protein. They do not, however, contain any detectable amounts of deoxyribonucleic acid. Many cytoplasmic enzymes can be detected in these small cells, as well as in the parental population. With the aid of Dr. Jane Setlow, we have shown that the photoreactivating enzyme, although present in the parental cells, is not detectable in the small cell.

The genetic locus involved in this mutation is very close to the "lon" locus previously described as playing an important role in the control of cell division. The mutant strain is also unusually resistant to ionizing radiation. However, genetic analysis of recombinants obtained from a cross with nonmutant donors suggests that the radiation resistance is associated with a locus near, but not identical to, the one controlling the unusual division behavior.

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7.5 GENETIC TRANSFER TO MINICELLS OF *ESCHERICHIA COLI* K-12

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Roy Curtiss III

The mutant strain 54 of *Escherichia coli* K-12 produces minicells which are about one-tenth the mass of normal *E. coli* cells and which contain RNA and protein but no DNA.[2] Since strain 54 is an F^- recipient, it was feasible to determine whether genetic transfer by conjugation to the minicells could take place. If such transfer could take place, then it would be possible to determine whether transcription and translation of the transferred information occurred. Also the system would provide the opportunity to isolate and characterize such genetic elements as the fertility factor F.

We first demonstrated by electron microscopy that F^+, F' *lac*$^+$, and Hfr donor strains would efficiently form specific pairs with F^- minicells separated from the parent cells by differential centrifugation. The paired donor and minicells were often at a distance from each other, being connected by a donor pilus. We also infected the minicell producing strain 54 with the fertility factor F and with an F' *lac*$^+$ factor. The minicells separated by differential centrifugation from the F^+ and F' *lac*$^+$ 54 strains occasionally possessed donor pili, but invariably these pili were attached backward as deduced by their inability to attach the donor-specific DNA phage, fl.[3] Specific pairs between F^+, F' *lac*$^+$, or Hfr donors and minicells from the F^+ or F' *lac*$^+$ 54 strains were infrequent.

By using F^+, F' *lac*$^+$, and Hfr donors whose DNA was labeled with ^{14}C-thymidine, it was possible to demonstrate physical transfer of TCA-insoluble genetic material to minicells from the F^- 54 strain. The amount of ^{14}C-thymidine label transferred to F^- minicells by F^+, F' *lac*$^+$, and Hfr donors was roughly equivalent. However, no physical transfer was detectable to minicells from either the F^+ or F' *lac*$^+$ 54 strains. Since most of these minicells from the F^+ and F' *lac*$^+$ 54 strains did not possess donor pili and did not form specific pairs with other K-12 donor strains, it is concluded that donor strains may possess a surface component, in addition to donor pili, which prevents pair formation with other donor cells. We plan to study this surface component further by immunological techniques.

Adler, Fisher, Cohen and Hardigree[2] have shown that the minicell-producing strain 54 can be induced

Fig. 7.4.1. An Electron Micrograph of *Escherichia coli* Undergoing an Abnormal Division That Yields One Normal Cell and One Small Cell Lacking Genetic Material.

to synthesize β-galactosidase and that minicells separated from a preinduced culture contain β-galactosidase. However, minicells separated from an uninduced culture cannot be induced to synthesize β-galactosidase. Thus, it was possible to determine whether minicells harvested from the F′ *lac*$^+$ minicell-producing strain or minicells having received the F′ lac$^+$ by conjugation could be induced to synthesize β-galactosidase. In both cases there was no induced synthesis of β-galactosidase. Thus, it can be concluded that either the lac_z gene cannot be derepressed, or if derepressible cannot be transcribed, or if transcribed the message cannot be translated.

Much further work with this system is anticipated, and present efforts are being directed toward isolating the fertility factor F by the Klineschmidt technique. We hope to show in the electron micrographs that F is circular and, based on its length, to calculate its molecular weight.

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7.6 ENERGY REQUIREMENTS FOR SPECIFIC PAIR FORMATION DURING CONJUGATION IN *ESCHERICHIA COLI* K-12

Roy Curtiss III D. R. Stallions

Fisher[1] concluded that specific pair formation, the first step during bacterial conjugation, was an endergonic process demanding the utilization of a metabolizable carbon source. This conclusion has been reiterated without challenge by Jacob and Wollman[2] and by Hayes,[3] even though it was not until 1962 when de Haan and Gross[4] defined specific pair formation as a union between donor and recipient cells resistant to dilution. The realization that Fisher[1] did not test the energy requirements during specific pair formation and the knowledge that donor cells are preendowed with appendages, called donor pili, which are responsible for specific pair formation,[5,6] caused us to experimentally determine the energy requirements, if any, during specific pair formation.

Our experiments made use of the fact that NaCN at 2×10^{-3} *M* reversibly inhibits electron transport. Thus, we took Hfr and F$^-$ parents which had been treated with 2×10^{-3} *M* NaCN for either 0, 1, or 15 min and mated them either in the presence or absence of NaCN for 5 min to allow for the formation of specific pairs. Following this time for pair formation the mating mixtures were diluted 1:1000 to prevent further pair formation and to allow reversal of the cyanide inhibition. The recombination frequencies obtained for each 60-min mating were the same except for the matings kept in 2×10^{-3} *M* NaCN throughout; in this last case a 20-fold reduction in recombinant yield was observed. Similar experiments using 10^{-3} *M* 2,4-dinitrophenol were also conducted with similar results. These and other results led us to conclude that specific pair formation during bacterial conjugation is not an endergonic process.

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7.7 MACROMOLECULAR SYNTHESES AND ENERGY METABOLISM DURING CONJUGATION IN Hfr × F$^-$ MATINGS OF *ESCHERICHIA COLI* K-12

Roy Curtiss III Jane A. Mays
D. R. Stallions

The need for DNA, RNA, or protein synthesis or for energy metabolism by either conjugating parent was determined with Hfr and F$^-$ mutants unable to

synthesize thymine, uracil, or amino acids or to utilize given carbon sources.

We first determined the effects of preventing DNA, RNA, or protein synthesis or energy metabolism in either or both parents by measuring inhibitions in recombinant yield after 30 to 50 min of mating. When inhibitions in recombinant yield were observed, interrupted matings were performed to determine whether the inhibition was due to an alteration in the rate of chromosome transfer or to a lessening of the number of cells capable of chromosome transfer. When the latter effect was noted, we determined whether the synthetic activity was required during the early stages of mating or throughout. To do this, Hfr and F^- bacteria were mated for 5 min in the presence of the needed metabolite and then diluted 1:200 into medium with or without the metabolite.

The conclusions, drawn from results of several hundred matings, are as follows: (1) Prevention of DNA, RNA, or protein synthesis in either or both parents does not alter the rate of chromosome transfer, although the Hfr parent normally replicates its chromosome during transfer. (2) The Hfr parent needs protein synthesis, DNA synthesis, and energy metabolism during the first 5 min of mating to allow all the cells to participate. (3) Prevention of DNA or protein synthesis in the F^- parent is without effect on the frequency of recombination. (4) The inability of the F^- parent to utilize the supplied carbon source results in a reduced rate of chromosome transfer, whereas the inability of the Hfr parent to utilize the supplied carbon source does *not* alter the rate of chromosome transfer.

Based on these and other studies the following sequence of events during bacterial conjugation is postulated. (1) Specific pair formation occurs with no metabolic requirements.[1] This union is mediated by the presence of donor pili on the Hfr parent.[2,3] (2) Protein synthesis is required in the Hfr parent (F initiator) to allow chromosome mobilization, which is the production of a chromosome that can be transferred linearly. (3) DNA synthesis is then required in the Hfr parent to propel the chromosome through the conjugation tube. From studies by Curtiss and Caro,[4] it is believed that the donor pilus also acts as the conjugation tube. The need for protein and DNA syntheses in the Hfr parent during steps 2 and 3 requires active energy metabolism. (4) Further chromosome transfer requires homologous pairing between the lead region of the Hfr chromosome and the comparable portion of the F^- chromosome.[5] (5) The F^- parent "winds in" the donor chromosome at a constant rate, ensuring orderly inheritance of transferred genetic material. This F^- conjugal activity is dependent on energy metabolism and independent of DNA, RNA, and protein syntheses.

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7.8 MECHANISM OF CHROMOSOME MOBILIZATION AND TRANSFER BY F^+ DONORS OF *ESCHERICHIA COLI* K-12

Roy Curtiss III Jane A. Mays[1]
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Integration of the fertility factor F into the circular chromosome of F^+ donors to allow chromosome mobilization and transfer is not necessary. The formation of Hfr mutants by this mechanism accounts for less than 5% of the recombinants formed in $F^+ \times F^-$ matings.[3] In studying the principal mechanisms for chromosome mobilization and transfer by F^+ donors, we have shown that (1) there are some F^+ cells in the population which can transfer any given genetic marker soon after commencement of mating; (2) F^+ donors transfer their chromosomes linearly at the same rate as Hfr donors; (3) F^+ mutant donors, with linear chromosomes, transfer markers from either end in an oriented manner; (4) completion of vegetative chromosome replication is necessary before an F^+ cell can transfer its chromosome; (5) protein synthesis is required for chromosome mobilization, which can be equated to the linearization of the intertwined fully replicated chromosomes; and (6)

DNA synthesis is not required during chromosome transfer.

These observations are interpreted by a sequence of events as follows: (1) Vegetative chromosome replication in the F^+ donor must go to completion, resulting in intertwined circular daughter chromosomes; (2) protein synthesis is then necessary to allow separation of the daughter chromosomes to produce two temporarily linear chromosomes (note that this step is equivalent to the initiation of a new round of vegetative chromosome replication); (3) if the F^+ cell is then paired with an F^- recipient, there is a low probability that one of the linear F^+ chromosomes will be transferred without further replication; and (4) by effective homologous pairing between the lead region of the F^+ chromosome transferred and the comparable portion of the F^- chromosome, the F^- parent controls the rate of chromosome transfer by winding in the F^+ chromosome. This last postulate is based on results obtained by Curtiss and Charamella.[4] The breaks in the circular F^+ chromosomes must be randomly distributed among the cells in the population. Thus, this model of chromosome mobilization and transfer is compatible with, but does not prove, the theory that the origins for initiation of vegetative chromosome replication are randomly distributed about the genome.

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7.9 LOCALIZATION OF GENES SPECIFYING tRNA SPECIES IN *ESCHERICHIA COLI* K-12

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The purpose of this recently initiated study is to develop techniques for isolation of specific gene fragments from *Escherichia coli* K-12. In order to do this, F′ elements will be transferred into appropriate mutant derivatives of *Proteus mirabilis* by conjugation. Since *E. coli* DNA has a 50% G-C content and *P. mirabilis* DNA a 38% G-C content, no genetic exchange can 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 isolated *E. coli* DNA, which is enriched for a specific segment of the genome, will then be tested for ability to hybridize with *E. coli* tRNA. Results of these mapping experiments will determine the feasibility of other experiments such as in vitro synthesis of biologically active tRNA from a DNA template, determination of whether *all* tRNA's coding for the same amino acid are necessary, etc.

Presently we have isolated and characterized numerous auxotrophic mutants of *P. mirabilis* which were induced by uv or nitrosoguanidine. This work is continuing in order to get a more complete collection of mutants containing mutations in all parts of the chromosome.

Simultaneously we are adding to our collection of F′ factors by employing the F′ isolation procedure developed by Berg and Curtiss.[1] In doing this we are using many different Hfr strains with different points of origin in hopes of eventually getting a series of overlapping F′ factors so that every portion of the K-12 genome will be contained on at least one F′ factor. We now have a series of nine different and overlapping F′ factor types containing from as little as 0.4 to 10% of the K-12 genome. These F′ factors collectively contain all the information from the *ara* to *gal* loci, which is about 20% of the total genome. Experiments to determine optimal conditions for the conjugal transfer of these F′ factors into *P. mirabilis* have just begun.

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Radiation Immunology

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8.1 VARIATION IN BLAST CELL CYCLE DURING LOG-STATIONARY PHASE OF SECONDARY ANTIBODY RESPONSE INITIATED IN IN VIVO DIFFUSION CHAMBER CULTURES

Toshihiko Sado

Introduction. – Earlier studies in this laboratory showed that the ^{3}H-thymidine index[1] [the ratio of the fraction of cells in the DNA synthesis (*S*) phase to the total cell population] of immature antibody-forming cells cultured in in vivo diffusion chambers changed drastically during the log-stationary phases of secondary antibody response. Such variations could have resulted from: (*a*) a change in the duration of *S* phase, (*b*) a change in the generation time, (*c*) a change in the total number of cells constituting the cell population under examination, or (*d*) a combination of two or more of these variables. Because of its significance to the process of suicidal differentiation of antibody-forming cells, experiments were carried out to estimate the mitosis time as well as generation time according to the colchicine method of Leblond.[2]

Methods. – Chambers containing 24×10^6 primed spleen cells and the test antigen (24×10^5 sheep RBC) were implanted into the peritoneal cavity of sublethally irradiated isologous recipients. At various intervals after culture, a group of chamber-

bearing mice was injected with colchicine (0.15 mg/mouse) intraperitoneally. Two hours later chamber contents were recovered, cell smears were prepared, and the frequency of blast cells in metaphase was determined. Another group of mice that received no colchicine served as untreated controls, and smear preparation from this group was used to determine the normal frequency of mitosis of blast cells.

Results and Discussion. – From the data given in Table 8.1.1, the mitotic (M) and generation time (GT) were calculated using the equations $M = Pt/Q$ and $GT = 100M/P$, respectively, where P is the normal frequency of mitosis and Q represents the frequency of blast cells in metaphase observed t hr after the administration of colchicine. From the table it can be seen that GT of blast cells was estimated to be as short as 4.5 hr on day 3 after antigen stimulation. Between days 4 and 6 it was approximately 8 hr and on day 9 it was as long as 18 hr. The estimated GT of 8 hr between days 4 and 6 is consistent with our earlier estimate based on waves of labeled mitosis.[3] In the control group, where the isologous mouse RBC rather than sheep RBC was introduced in the chamber cultures, the GT of blast cells was two times longer than that observed in the antigen-stimulated group on day 3 and was comparable on day 9.

The total number of blast cells changed during the period of observation, and this could affect the interpretation of the results. However, we feel that this variable is not a significant factor here because the interval between the time of injection of colchicine and the time of sampling was so short that any possible change in the total number of blast cells should be minimal. Experiments are now in progress to study the variation in duration of S phase using double-labeling autoradiography utilizing ^{3}H- and ^{14}C-thymidine.

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8.2 CHARACTERIZATION OF ANTIBODY-SYNTHESIZING CULTURES

D. C. Vann

Introduction. – Investigations of the subcellular mechanism and regulation of antibody synthesis can be greatly facilitated through the use of well-characterized cultures of lymphoid cells which produce high levels of specific antibodies. The present studies were carried out to determine the conditions for the production and characterization of such cultures.

Methods and Results. – Previous work[1] has shown that diffusion chamber cultures of antigenically stimulated spleen cells from primed mice

Table 8.1.1. Cell Cycle Time of Blast Cells Undergoing a Secondary Anti-Sheep-RBC Response in in Vivo Diffusion Chamber Cultures

Group	Time After Culture (days)	Normal Frequency of Mitosis (P) (%)	Frequency of Colchicine-Blocked Metaphase (Q) (%)	Mitotic Time (M) (hr)	Generation Time (GT) (hr)
Ag-stimulated	3	129/1138 (11.34)	223/514 (43.85)	0.517	4.56
	4	68/1159 (5.87)	184/739 (24.9)	0.471	8.02
	5	49/636 (7.70)	58/256 (22.6)	0.681	8.84
	6	17/403 (4.20)	42/178 (23.6)	0.356	8.48
	9	9/676 (1.30)	36/316 (11.4)	0.230	17.69
Control	3	51/645 (7.90)	133/593 (22.89)	0.690	8.73
	9	23/390 (6.20)	139/1109 (12.53)	0.990	15.96

incorporate ^{14}C-labeled amino acids into gamma globulins when removed from their hosts and cultured in vitro for short periods of time. Chambers containing 2×10^8 primed spleen cells and 2×10^7 sheep red blood cells, the immunizing antigen, or 2×10^7 mouse red blood cells, nonstimulated controls, were implanted in sublethally irradiated recipient mice. Six days after implantation the chambers were removed from their hosts and wiped clean with sterile gauze. The chambers were then opened with a razor blade and placed in culture medium containing 10 $\mu c/ml$ ^{14}C algal protein hydrolysate without further manipulation. Samples were taken after increasing periods of culture at 37°C. Incorporation of ^{14}C amino acids into total protein was followed by the filter paper disk method. Label in γG globulin was measured by specific precipitation with rabbit antimouse γG globulin, and label in specific antibodies was determined by binding to stroma of sheep erythrocytes.[2] Radioimmunoelectrophoresis and sucrose-gradient centrifugation analysis of the total culture media and eluates from the stroma were used to characterize the labeled materials.

It was found that the kinetics of appearance of TCA-precipitable radioactivity was essentially linear for the first 6 to 8 hr and tended toward plateau values by 20 to 24 hr. More than 85% of the counts which were precipitable by antimouse γG globulin would also bind to sheep stroma. The increase in incorporated label in cultures which received antigenic stimulation over those which did not was identified as γG globulin with a sedimentation value of approximately 7S by radioimmunoelectrophoresis and sucrose-gradient centrifugation. Evidence obtained from experiments in which identical amounts of radioactive algal protein hydrolysate were mixed with culture medium supplemented with 20% mouse serum to serve as a cell-free control indicated an association of labeled material with some of the preexisting proteins. This binding occurred primarily with albumin and α globulin and was not significant with γ globulins. These difficulties can be overcome through the use of selected labeled amino acids.

Discussion. – These findings indicate that specific antibody synthesis by diffusion-chamber cultures of lymphoid cells can be monitored in vitro. The conditions of culture can be varied in a variety of ways which may provide insight into the regulation of antibody synthesis at the subcellular level.

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8.3 SYNTHESIS OF ANTIBODY BY SPLEEN CELLS AFTER EXPOSURE TO KILOROENTGEN DOSES OF IONIZING RADIATION

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Toshiteru Morita

Introduction. – During the height of antibody response, most fully differentiated, mature antibody-synthesizing cells disappear randomly with a very short half-time of about two days. Previous cytokinetic studies[2] on antibody formation and on the effects of physical and chemical insults on immune competence indicated to us that these cells disappear not because of "old age" but because they are "forced to" by oncoming waves of newly differentiated antibody-synthesizing cells. We thus reasoned that mature antibody-synthesizing cells should persist for a longer length of time, provided that proliferating precursor cells were eliminated immediately after the first wave of mature antibody-synthesizing cells appears. Accordingly, mouse spleen cells in diffusion chambers were studied in an effort to assess the radiation survival curves of lymphoid cells during the different serologic phases of immune response and to characterize morphologically and metabolically those radiation resistant cells.

Results and Discussion. – The results showed that the capacity of immunocompetent progenitor cells to proliferate and differentiate into antibody-synthesizing cells was highly sensitive to x irradiation. The D_0 assessed serologically in terms of plateau titers was 209 r (95% confidence limits of 161 r and 251 r) and an extrapolation number of 2.17 (95% confidence limits of 1.37 and 3.43). The fully differentiated progenies, which were made up mainly of mature plasma cells, were resistant. As judged by serological and isotope incorporation studies these radioresistant cells were able to synthesize antibody effectively for as long as several days after their exposure to radiation doses up to 10,000 r.

As a result of these studies a method with a high recovery yield was devised for obtaining a highly purified preparation of dispersed monospecific antibody-synthesizing cells. This was done by simply exposing primed spleen cells to 10,000 r at the end of the log phase of response and harvesting the culture several days later.

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8.4 THE INVOLVEMENT OF THE SPLEEN IN SENESCENCE AND DEATH IN AN INBRED STRAIN OF MICE

J. F. Albright Takashi Makinodan

Introduction. – There is a steady decline in the number of immunologically competent cells in the senescing animal[1] and it has been argued[2] that depletion of these cells is causally related to aging and death. During attempts to immunologically rejuvenate senescent animals by infusing spleen cells from syngeneic donors, it was observed that when the donors themselves were old animals, the recipients died at a considerably faster rate than they did lacking spleen cell "therapy." In order to learn more about the cause of such hastened death, experiments were performed that included assessment of the weights of various organs – particularly the spleen – at the time of death. Some of the data are presented here.

Methods and Results. – Mice of the $BC3F_1$ strain (C57BL ♀ × C3H ♂) F_1 were caged in groups of ten. Several hundred mice of age 76 weeks were employed in the experiments. They were divided into six groups, five of which were exposed to 400 r x irradiation. After irradiation, mice in four of the groups were injected intravenously with spleen cells from syngeneic donors of various ages as outlined in Table 8.4.1. Mice of a sixth group remained unirradiated and served as normal, untreated controls. After treatment the mice were housed five per cage and observed at regular intervals until all were dead. The time at which 50% of the mice in a given group had died is recorded in the table. Whenever possible mice were removed shortly after death and autopsied. The means of body weights and spleen weights are recorded in Table 8.4.1. It is clear that the time at which 50% of the recipients of spleen cells had died was related to the age of the donors and the degree of enlargement of the donor spleen. The pronounced enlargement at the time of death of the spleens of recipients of cells from aged donors is obvious.

Discussion. – We assign considerable significance to splenic enlargement during senescence as a clue to the cause(s) of death. Immunologically competent cells are greatly depleted in such spleens in old mice.[2] Whether or not such spleens are cancerous, and whether the factor stimulating enlargement is cellular or noncellular, will be determined.

Table 8.4.1. Shortening of Mean Life Span and Concomitant Splenic Enlargement of Recipients of Spleen Cells from Aged Syngeneic Donors

X-Ray Exposure (r)	Number of Donor Spleen Cells	Age of Donors (weeks)	Time of 50% Death (weeks)	Autopsy Data	
				Mean Body Weight (g)	Mean Spleen Weight (mg)
0			128	26	427[a]
400			128	25	328
400	10^8	12	132	24	453
400	10^8	76	114	30	458
400	10^8	100	96	36	1,103
400	5×10^7[b]	100	91	36	1,750

[a]Mean spleen weight of normal, 100-week-old controls = 125 mg.
[b]From enlarged spleens (mean, 328 mg).

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8.5 PRIMARY AND SECONDARY ANTIBODY RESPONSE BY MICE AND THEIR SPLEEN CELLS TO HUMAN GAMMA GLOBULIN

A. E. Sassen E. H. Perkins

Introduction. – The cytokinetics of antibody production in response to particulate antigens (heterologous red blood cells) have been extensively studied in the intact mouse, the cell transfer system, and the diffusion chamber culture system. However, a comparable study in response to a soluble antigen is lacking. The present work was therefore undertaken to determine the efficacy of human gamma globulin (HGG), a simple soluble protein, as an immunogen in the intact mouse and in the cell transfer and diffusion chamber culture systems.

Results. – Heat-aggregated HGG proved to be a potent antigen. The size of the aggregates did not seem important, as soluble aggregates were as effective as insoluble aggregates. Aggregated HGG induced (*a*) an excellent primary and secondary response in the intact mouse, (*b*) a poor primary but an excellent secondary response in the cell transfer system, and (*c*) was ineffective in inducing a primary response but induced a good secondary response in the diffusion chamber culture system.

As expected, antibody response was antigen-dose-dependent, but the effective dose of antigen was broad; that is, a maximum primary response in an intact mouse and a maximum secondary response in the cell transfer and diffusion chamber culture systems could be induced with aggregated HGG in doses ranging from 10^{-5} to 10^{-7} g. A dose as low as 10^{-12} g was still effective in inducing a secondary response in intact mice and in the cell transfer system. When the mice were primed with aggregated HGG an excellent secondary response was obtained even with use of nonaggregated HGG. At the limiting antigen dose level (10^{-11} to 10^{-12} g) it was not possible to deduce with certainty which form was superior.

Discussion. – The results presented here show that HGG is an excellent immunogen. Thus, antibody response was detected with doses as low as 10^{-9} to 10^{-12} g. This means that it is comparable, if not superior, to flagellen antigen, the most potent protein antigen known to date. Studies are now in progress to assess the cellular manifestations of immune response using HGG as the test antigen.

8.6 ERYTHROPOIETIC POTENTIALITIES OF CELLS OF THE EMBRYONIC YOLK SAC

J. F. Albright[1] Julia W. Deitchman

Introduction. – During ontogeny of the mouse and other mammals the erythrocytes that appear initially are generated by the yolk sac. These erythrocytes are readily distinguished from those of the adult because they are 3 to 4 times as large and are nucleated. We are interested in the progenitor cells of the yolk sac that give rise to this "primitive" series of yolk sac. Specifically, we wished to determine whether or not the progenitor cells could, under proper conditions, generate adult-type, definitive erythrocytes.

Methods and Results. – Yolk sacs were surgically removed from nine-day-old fetuses of C57BL/6 mice. The yolk sacs were disrupted, the cells dispersed and counted. The dispersed yolk sac cells were placed in diffusion chambers (2 to 5×10^6 cells/chamber), and these preparations were implanted into the peritoneal cavities of irradiated adult culture mice. At intervals during culture, chambers were removed, the cells recovered and counted, and slides prepared for evaluating the proportions of cells of various types, particularly of primitive (embryonic type) and definitive (adult-type) erythrocytes. It was found that during culture in the irradiated adult environment, the yolk sac progenitors rapidly ceased production of primitive erythrocytes and initiated production of definitive erythrocytes.

Discussion. – The results suggest that the differentiative pathway of erythrocyte progenitors in the yolk sac may be altered under the influence of factors present in the adult environment. The mechanism(s) that are subject to modification of expression may include those involved in the production of specific macromolecules that characterize the erythrocyte.

References

[1]Visiting investigator during the summer of 1966 at The Jackson Laboratory, where part of this work was performed.

8.7 SHEEP STROMA AS AN IMMUNOABSORBENT

R. A. Brown W. R. Somers
Takashi Makinodan

Introduction. – There is currently a widespread interest in immunoabsorbents. Most, if not all, of this work has had as a goal the purification of antibodies directed against a well-defined antigen using hyperimmune sera. These immunoabsorbents are in general ill suited for work with early immune sera because of high "noise" levels and a low affinity for the antibody. This is a report of the successful use of sheep stroma as an immunoabsorbent for early primary and secondary sera.

Experimental. – Stroma was prepared by lysis of red blood cells in hypotonic saline and extensive washing to remove hemoglobin. Mice were immunized by injecting sheep red blood cells or stroma and the mice bled at appropriate times. In some experiments the mice were injected with ^{14}C amino acids 4 to 6 hr prior to bleeding. Antibody was adsorbed to the stroma for 2 hr at 25°C and 16 hr at 4°C. After washing the stroma with cold saline, the antibodies were eluted at low pH and then the pH of the supernatant brought to 7. The purified antibodies were centrifuged on a sucrose gradient and the gradients analyzed either in a spectrophotometer using a flow cell or in a scintillation counter using paper disks. Selected cuts containing pure macroglobulin were analyzed by short-column equilibrium sedimentation.

Results. – Data obtained both by monitoring the ultraviolet absorption of the sucrose gradients and ^{14}C activity would indicate that antibodies with a purity of 95 to 99% can be obtained by elution from stroma after extensive washing. Recovery is of the order 25 to 50%. By the use of less extensive washing the recovery of antibody can be doubled with some sacrifice in purity.

At five days in the primary response there is approximately three times more 7 *S* antibody than there is 19 *S* antibody. This is contrary to what is found by means of hemagglutination; but it is probable that hemagglutination detects only a limited class of antibodies.[1]

Macroglobulin antibodies obtained by this technique are homogeneous in molecular weight with a value of 960,000. Further manipulation of the pure antibody (freezing and thawing or ultrafiltration) leads to degradation of the molecule. The lability of the purified molecule may explain the lower molecular weight values obtained by other investigators.[2]

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8.8 EFFECT OF X IRRADIATION ON RNA METABOLISM OF PHA-TRANSFORMED LYMPHOCYTES IN VITRO

Hiroo Yamamoto

Introduction. – Phytohemagglutinin (PHA) stimulates the transformation of human peripheral lymphocytes to blast cells in vitro. Many of these lymphocytes survive and transform under high levels of irradiation. This study was designed to define the relationship between RNA synthesis and blastogenesis in both irradiated and nonirradiated PHA-stimulated lymphocytes. To this end a 22-hr assay system has been devised. Purified lymphocyte suspensions were prepared from normal heparinized blood. PHA was added as stimulant to the culture containing ^{14}C-uridine. RNA was extracted by successive suspensions and centrifugations in trichloroacetic acid and ethanol-ether and the radioactivity determined in a scintillation counter. Under the culture conditions of the assay system, there was no synthesis of DNA as measured by incorporation of ^{14}C-thymidine.

Results and Discussion. – RNA synthesis rose rapidly in the initial 24 hr, began to level off by 72 hr, and dropped off by 96 hr in stimulated cultures. The maximal dose response to PHA was established with 0.01 to 0.005 unit. To elicit maximal RNA synthesis PHA was continuously required during the assay period of 22 hr. RNA synthesis increased linearly with increase in the number of lymphocytes from 1×10^5 to 2×10^6 cells/ml. Cells from different normal donors responded similarly to PHA.

The determination of the classes of synthesized RNA is currently under investigation.

Mammalian Genetics

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9.1 EFFECT OF THE INTERVAL BETWEEN X IRRADIATION AND CONCEPTION ON MUTATION FREQUENCY IN FEMALE MICE

W. L. Russell Elizabeth M. Kelly

Recently published results on female mice exposed to fission neutrons show that the interval between irradiation and conception has a tremendous effect on mutation frequency.[1] Conceptions in the first seven weeks after irradiation with approximately 63 rads yielded 59 specific locus mutations in 89,301 offspring. After that, no mutations were found in a total of 120,483 offspring.

It was obviously important to find out whether the result was a peculiarity of neutron irradiation

or a general radiation effect. Early work with gamma irradiation at low dose rate suggested that the phenomenon might be general, but the data were not extensive enough to be statistically conclusive.[2] Experiments with x rays were accordingly started, despite the handicap of having to work with a low dose (50 r). With higher doses, at the dose rate used (81 r/min), the females become sterile too soon after irradiation. This difficulty was greatly reduced in the neutron work because the relative biological effectiveness for mutation induction was much higher than that for effects on fertility. An additional handicap was discovered during the x-ray experiment which was, however, of great importance in itself. This was the finding that the mutation frequency in the early mating period was lower than expected from the frequencies at higher doses on the assumption of a straight-line relation between mutation frequency and dose. This has been discussed in an earlier report.[3]

The extensive data so far collected to overcome these difficulties are presented in Table 9.1.1. They have reached the point where the mutation frequency in the later period is significantly below that in the early period ($P = 0.026$). Thus, the phenomenon appears to be general and, therefore, of much greater importance than if it had been solely a neutron effect.

The cause of the effect presumably lies in the nature of the oocyte follicle stages involved. The early period is long enough for the conception of two litters. The low mutation frequency in the later period must have come from oocytes that were in less mature follicle stages at the time of irradiation. It is not yet known whether the marked difference in mutation frequencies is due to a low mutational sensitivity of oocytes in early follicle stages, to an efficient repair mechanism in these stages, or to cell selection. The accompanying report by Oakberg *et al.*, which reveals a high metabolic activity of oocytes in early follicle stages and a dramatic decrease in activity in later stages, strengthens the possibility that repair is involved.

Table 9.1.1. Frequency of Specific-Locus Mutations at Two Intervals After Exposure of Female Mice to a Dose of 50 r of 81 r/min X Irradiation

Interval Between Irradiation and Conception	Number of Offspring	Number of Mutations at Seven Loci
Up to seven weeks	145,585	11
More than seven weeks	57,538	0

Whatever the cause, it is extremely effective. It was pointed out[1] that in the neutron data even the upper 99% confidence limit of the zero mutation frequency in the later period is lower than the spontaneous rate in male mice. Almost half as many more offspring in this period have now been added by the x-ray experiment, and still no mutations have been observed.

Application of this result to the estimation of genetic effects in women should be made with great caution until more is known about the comparability of the oocyte stages involved in the two species. Some information might be obtained by analyzing the genetic data from human radiation exposures in terms of the time interval between irradiation and conception.

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9.2 CHEMICALLY INDUCED DOMINANT LETHALS IN MALE MICE[1]

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Compounds with known specific mutagenic action in microorganisms were tested for the induction of dominant lethals in male mice. Methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) are both alkylating compounds which react with the bases in the DNA in the following decreasing order: guanine > adenine > cytosine. The tertiary phosphate groups of the sugar-phosphate backbone of the DNA are also alkylated but to a smaller extent than the bases; there is no indication that this latter reaction results in mutations. The alkylated bases may induce base-pair transitions by

erroneous pairing, or they may be deleted from the DNA leaving a "gap" in the sequence of the bases. There exists a comparable probability that any base can be incorporated opposite to a gap, which will result in a base-pair transition, transversion, or restoration of the original base-pair sequence. Gaps might also lead to single base-pair deletions or cross-linkage of the DNA. Evidence from studies in phage and bacteria indicates that cross-linkage of the DNA gives rise to larger aberrations. Since methylated purines have a greater chance than ethylated purines to be deleted from DNA, MMS should be more effective than EMS to induce chromosome aberrations. In phages the base-pair transitions induced by MMS and EMS are preferentially from GC to AT. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) induces in Neurospora mainly or exclusively base-pair transitions predominantly in the direction from AT to GC.[3] If the induced genetic alteration in mammals is similar to that in microorganisms, one would expect a high frequency of dominant lethals after treatment with MMS, an intermediate frequency after treatment with EMS, and a very low frequency after treatment with MNNG. This prediction was tested in the following experiment.

Hybrid male mice, $(101 \times C3H)F_1$, 10 to 12 weeks old, were injected intraperitoneally with a single nontoxic dose of the test compound (Table 9.2.1), or with an equal volume (1 cm^3) of Hanks's solution. Immediately following injection each male was caged separately with two $(101 \times C3H)F_1$ females, 34 to 37 weeks old. A second and a third mating were made after 7 and 14 days, respectively, at which time each male was transferred to a fresh set of two females. This transference of males separates gametes treated as spermatogonia or late and early spermatids.[4] Matings were detected by daily examination of females for vaginal plugs. The uterine contents of fertilized females were examined 14 to 17 days postconception. The induction of dominant lethals was calculated in the following way:

$$100 - \frac{\text{embryos experimental group}/♀ \times 100}{\text{embryos control group}/♀}.$$

If the treatment is ineffective the value for dominant lethals will be zero in an ideal sample size. The range of the variability in these experiments is indicated by small deviations in both directions. In Table 9.2.2 only positive values are given.

The effects of the treatment on the fertility of males, the percentage of dead implants, and the induction of dominant lethals are summarized in Table 9.2.2. Fertile matings 1 to 14 days after injection of MMS are significantly below the incidence of fertile matings in the control group. The treatment with EMS does not affect the frequency of fertile matings. The percentage of fertile matings 1 to 7 days after treatment with MNNG is lower than in the corresponding control group. The *P* value for this difference is 0.06.

In aging virgin females the frequency of total implants decreases, the frequency of dead implants increases. Hybrid virgin females 34 to 37 weeks old have about 17% fewer implants than 10- to 12-week-old hybrid females. The number of dead implants is twice as high in older females as in younger.[5] This observation accounts for the high frequency of dead implantations in the controls of Table 9.2.2. After treatment with MMS the frequency of dead implants is significantly higher than in the control group. In only one mating group, 8 to 14 days after injection of 200 mg of EMS per kilogram of body weight, was a significant increase of dead implants over the controls observed. The injection of MNNG had no effect on the frequency of dead implants.

The injected doses of MMS induced more dominant lethals in spermatozoa and late spermatids than the irradiation of hybrid male mice with 1000 r.[5] The frequency of dominant lethals after injection of 200 mg of EMS per kilogram of body weight in late spermatids is similar to the effect of 600 r of x rays.[5] The highest frequency of dominant lethals after irradiation is induced in early sper-

Table 9.2.1. Toxicity of Compounds in Male Mice

Compound	Dose (mg/kg)	Number of males	Survival (%)
MMS	300	6	0
	200	4	50
	150	14	100
EMS	500	6	67
	400	6	100
	300	6	100
MNNG	100	6	50
	75	6	67
	50	30	100

matids. Contrary to the x-ray effect, the early spermatids are less susceptible to the induction of dominant lethals after injection with MMS or EMS than the spermatozoa and late spermatids. No induction of dominant lethals was observed after injection of 50 mg/kg of MNNG.

The results of our experiments, summarized in Table 9.2.2, are in remarkable agreement with the prediction of the effect of the chemicals based on work with microorganisms. This conclusion supposes that the injected chemicals reach the testes. In an experiment with a negative result, for example, after injection of MNNG, we have until now no proof that the supposition is correct. However, it should be noted that the percentage of fertile matings 1 to 14 days after injection of MNNG is lower than the corresponding control matings.

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[1]Research sponsored in part by the AEC-NIH Molecular Anatomy Program.

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9.3 ANALYSIS OF GENETIC CHANGES IN A SHORT REGION OF LINKAGE GROUP II OF THE MOUSE

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Detailed analysis of a short region of a mouse chromosome has been made possible by the availability of a large number of independent genetic alterations in that region. A variety of mutation-rate experiments carried out over several years has utilized the closely linked markers *d*, dilution, and *se*, short ear (recombination, 0.16%) on linkage

Table 9.2.2. Induction of Dominant Lethals in Male Mice

Compound	Dose (mg/kg)	Dose (mM/kg)	Mating Period (days)	Number of Females	Fertile Matings (%)	Number of Implants	Number of Embryos	Dead Implants (%)	Induced Dominant Lethals[a] (%)
MMS	Control		1–21	36	77.8	240	214	10.8	0
			1–7	24	20.8	22	7	68.2	81.7
	150	13.62	8–14	24	0				
			15–21	24	87.5	152	124	18.4	22.7
			1–7	24	25.0	22	5	77.3	89.1
	100	9.08	8–14	24	29.2	22	11	50.0	79.4
			15–21	24	87.5	158	129	18.4	19.6
EMS	Control		1–21	36	83.3	247	213	13.8	0
			1–7	24	75.0	146	120	17.8	6.1
	200	16.11	8–14	24	83.3	142	77	45.8	45.8
			15–21	24	91.7	186	168	9.7	0
			1–7	24	83.3	173	158	8.7	0
	100	8.05	8–14	24	83.3	163	135	17.2	4.9
			15–21	24	87.5	179	160	10.6	0
MNNG	Control		1–21	36	80.6	252	212	15.9	0
			1–7	48	58.3	231	208	10.0	0
	50	3.40	8–14	48	64.6	267	239	10.5	0
			15–21	48	83.3	353	305	13.6	0

[a]See text for method of calculation.

group II. Mutant chromosomes recovered are those which in combination with the *d se* test chromosome give either *d, se,* or *d se* phenotypes. On the basis of complementation-test results, these mutants have now turned out to be of a variety of different types.

Materials. – Additional markers available in the region studied (but not included in the test stock) are *sv*, Snell's waltzer, and *tk*, tail kink, giving respectively 2 and 6% recombination with *d se*, and both on the same side. Mutants giving a *d* phenotype in combination with the *d se* test chromosome have, when tested, fallen into three broad classifications on the basis of their effect in the homozygote: d^x mutants are viable but usually darker than *d/d*; d^{op} mutants (which form the great majority of all *d* mutants) are dilute, with opisthotonic convulsions that prove lethal, generally by the time of weaning; d^{pl} mutants are prenatally lethal. Mutants giving an *se* phenotype in combination with the *d se* also have fallen into three broad classes: "*se*," homozygous viable (and generally indistinguishable from the test-stock marker); se^{pl}, homozygous lethal, prenatally; and se^{sv}, homozygous short ear and waltzer (only one found). Mutants giving a dilute, short-eared phenotype with the test-stock markers are for the most part deficiencies, prenatally lethal in homozygous condition. As outlined in the last report,[1] they fall into two broad groups on the basis of tests with *sv*, namely, Df (*d se*) and Df (*d se sv*). None of the deficiencies has included the *tk* locus.

Results. – Well over 300 combinations of mutants are being studied. Although tests are not yet complete, a number of interesting results have already emerged.

About 50 combinations of various *d* with various *se* mutants have so far yielded only wild-type (color, ears, behavior) with good viability. This includes d^{pl}/se^{pl}, d^{pl}/se^{sv}, d^{op}/se^{pl}, and d^{op}/se^{sv} genotypes.

Combinations of seven d^{pl} mutants with *sv* have all produced wild-type. However, of five different se^{pl}/sv combinations, one waltzes in a manner indistinguishable from *sv/sv*. (The remainder are wild-type.) It is concluded that this particular se^{pl} mutation is in fact Df (*se sv*) and that the order of markers must be *d se sv tk*. Tests show that the *se sv* deficiency does not include the *tk* locus.

Tests of dilute mutants with each other give the following results: d^{op}/d^{op} (36 combinations) and d^{op}/d^{pl} (seven combinations) have all produced the dilute opisthotonic phenotype. Of 21 d^{pl}/d^{pl} combinations set up, 12 have been prenatally lethal; and, surprisingly, two have been dilute opisthotonic (seven others not yet complete).

Tests of *se* mutants with each other yield the following results. Forty-five combinations of ten viables tested in all possible pairs gave no complementation for the ear phenotype. All of 20 "*se*"/se^{pl} and four se^{sv}/se^{pl} combinations made to date also were short-eared viable and nonwaltzing. Of ten se^{pl}/se^{pl} combinations now being produced, four, so far, have been prenatally lethal; two, surprisingly, have shown complementation for the lethal effect, being short-eared, completely viable. The combination se^{sv}/tk is wild-type.

Both the "short" and "long" *d se* deficiencies, that is, Df (*d se*) and Df (*d se sv*) types, respectively, have been tested with d^{op} mutants (18 combinations) and d^{pl} mutants (19 combinations). The phenotypes of d^{op}/Df are invariably dilute opisthotonic, and those of d^{pl}/Df are prenatally lethal. Similarly "*se*"/Df and se^{pl}/Df are short-ear viable and prenatally lethal, respectively, for both "short" and "long" *d se* deficiencies. As expected se^{sv}/Df (*d se*) is short-eared, nonwaltzer, while se^{sv}/Df(*d se sv*) is short-eared waltzer, with the waltzing behavior possibly more extreme than it is in se^{sv}/se^{sv}.

Conclusions. – The results outlined fit the complementation map (as yet incomplete) shown in Fig. 9.3.1. The relative positions of "d" and

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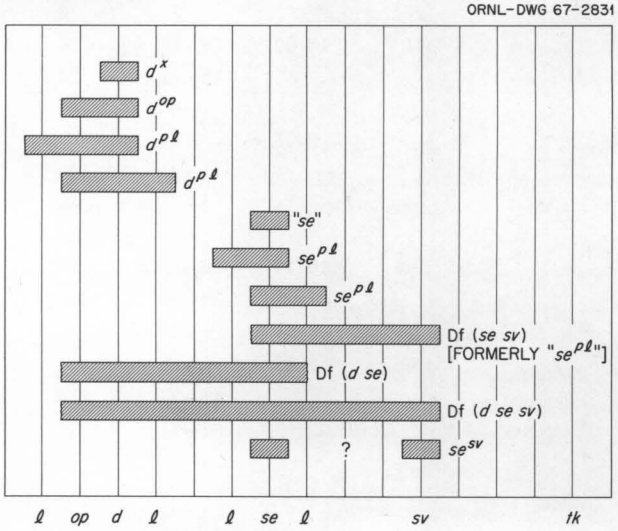

Fig. 9.3.1. Complementation Map for *d se sv* Region of Linkage Group 2 in the Mouse.

"op" are arbitrary. Work still in progress may further define the margins of the deficiency types and possibly yield subgroupings. The se^{sv} "mutation" best fits the assumption of a double event since it fails to meet various criteria for being an *se sv* deficiency. Recombination tests are in progress to test this assumption.

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[1]L. B. Russell, M. H. Steele, and H. M. Thompson, Jr., *Biol. Div. Semiann. Progr. Rept. July 31, 1966*, ORNL-3999, pp. 90–92.

9.4 ^{3}H-URIDINE LABELING OF MOUSE OOCYTES

E. F. Oakberg
Evelyn C. Lorenz
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Diane W. Slover

During preovulatory growth of the oocyte, a store of ribosomes, endoplasmic reticulum, nucleic acid, and possibly messenger RNA is accumulated in the cytoplasm for use in early cleavage divisions of the embryo. This suggested the use of ^{3}H-uridine as a method for timing oocyte development in the mouse, and for studying the metabolic activity of the growing oocyte.

Adult 101 × C3H hybrid female mice, eight weeks old, were injected intraperitoneally with 75 μc of ^{3}H-uridine and killed at intervals ranging from 15 min to 35 days after injection. Ovaries were fixed in Carnoy's acetic alcohol (3 : 1), embedded in paraffin, sectioned at 3 μ, and dipped in Kodak NTB_2 liquid emulsion diluted 1 : 1 with a 0.1% solution of Dreft. Exposures of six and eight weeks were used.

Oocytes were classified into eight stages on the basis of their follicular development: (1) only a few follicle cells, (2) a complete layer of flattened follicular cells, (3) a single layer of cuboidal cells, (4) two layers of follicle cells, (5) multilayered follicle, no antrum, (6) antrum forming, (7) single antrum of moderate size, and (8) mature Graafian follicle.

Degeneration of follicles owing to internal radiation from ^{3}H-uridine incorporated by follicle cells precluded the timing of oocyte development by this method. The changing pattern of ^{3}H-uridine with growth of the oocyte, however, was clearly revealed.

Labeling of both nucleolar and nonnucleolar regions of the nucleus occurred by 15 min and continued to increase in heaviness and in number of oocytes labeled until 1 hr after injection. A high level of labeling was maintained until 24 hr, after which frequency of oocytes with either nucleolar or nonnucleolar label decreased. Except for a few cells, cytoplasmic labeling did not appear until 12 hr and remained high for 15 days. Thus the general pattern of nuclear ^{3}H-uridine uptake by the nucleolus and nucleus and subsequent transfer to the cytoplasm as observed in a variety of other cells also occurs in the mouse oocyte.

The extent of ^{3}H-uridine incorporation, however, was related to stage of follicular development. Stage 1 oocytes had a turnover rate similar to that of ovarian stromal cells. Stages 2 to 4 showed heavy labeling which first appeared over the nucleus and later over the cytoplasm, where it persisted until the oocytes degenerated. The rate of incorporation slowed somewhat in stage 5, was very low in early stage 6, and stopped altogether in late stage 6 oocytes. Stage 7 and 8 oocytes never showed incorporation of ^{3}H-uridine.

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[1] and to mutation.[2] In a study on the effect of the interval between irradiation and conception on mutation frequency in female mice, Russell[2] observed that all mutations came from young conceived within the first seven weeks after irradiation, a period which usually is sufficient for the production of two litters. No mutations were observed in an even larger number of progeny conceived more than seven weeks after irradiation, that is, in young derived from oocytes that probably were in immature follicle stages at the time of irradiation. Russell[2] emphasized the importance of finding out whether the marked difference in mutational frequencies is due to a low mutational sensitivity of oocytes in early follicle stages, to an efficient repair mechanism in the immature oocyte, or to cell selection. The supply of late 6 and stage 7 and 8 oocytes, where metabolic activity is low, may be sufficient to give the first two post-irradiation litters, in which the mutation rate is high. Conversely, those litters conceived more than seven weeks after irradiation could have been derived from oocyte stages which were metabolically active at the time of radiation exposure. It

seems likely that metabolic activity would be closely correlated with capacity for repair. Thus the data on ^{3}H-uridine incorporation suggest that changing capacity for repair of genetic damage may be an important factor in the change in mutation frequency with time after irradiation as observed by Russell.[2]

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[2]W. L. Russell, *Proc. Natl. Acad. Sci. U.S.* **54**, 1552–57 (1965).

General Physiology

Radiation Biology	
G. E. Stapleton[a]	Carol M. Newman[b]
Molecular Physiology (10.1)	
W. D. Fisher	Tom Grizzard F. W. Shull, Jr. Rose Mary Dziak[c] Mary D. Murphy[c] A. W. Prestayko[d,e]

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10.1 THE CONTROL OF CELL DIVISION IN *ESCHERICHIA COLI* BY SUBSTANCES IN CELL EXTRACTS

W. D. Fisher H. I. Adler[1]
F. W. Shull, Jr.

We have shown previously[2–4] that cell-free extracts of *E. coli* can control cell division (cross-septation) in radiation-induced filaments of certain strains of *E. coli* such as AB1899MN. We are attempting to isolate and identify the active substances present in the cell extracts.

Results and Discussion. – The supernatant from high-speed centrifugation (150,000 × *g* for 300 min) is inactive in promoting division in irradiated cells unless a particulate fraction is added back to the supernatant.[2] However, a material which promotes cell division can be separated from an inhibitory material in the supernatant by ethanol fractionation at pH 4.5. The materials may also be separated by ion exchange resins; the stimulatory material is retained on cation exchangers and the inhibitory material on anion exchangers. From gel filtration data, the inhibitor and stimulator appear to have molecular weights of 500 to 1000.

As a working hypothesis, we propose that the inhibitory material may be a polycation and the stimulatory material a polyanion. Cell division would then be controlled by the balance of the two materials.

Current efforts are in two directions: (1) attempts at isolation and (2) testing of known compounds for biological activity on irradiated cells.

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Mammalian Recovery

C. C Congdon[a]

Secondary Disease (11.1–11.3)

C. C Congdon[a]
A. L. Kretchmar[b]
W. H. McArthur[b]
Kathleen H. Harrington
Alberta P. Henley
R. E. Toya, Sr.
L. W. Webb[c]
Mary S. Wedemeyer[d]

Recovery in Vitro (11.4)

L. H. Smith
T. W. McKinley, Jr.
B. S. Schnall[d]

Experimental Hematology (11.5)

Joan Wright Goodman
Gayle C. Bosma
Sarah G. Shinpock
Fay M. Martin[b]

Carcinogen Biology

C. C Congdon[a]
D. G. Doherty[a]
Frances E. Hacker

Animal Testing Laboratory (11.6–11.8)

M. L. Simmons[a]
C. B. Richter[a]
R. W. Tennant
D. T. Donald
J. A. Franklin
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11.1 THE EFFECT OF HYPOXIA ON MORTALITY FROM SECONDARY DISEASE

C. C Congdon M. L. Simmons
R. E. Toya, Sr.

Introduction. – In a study carried out by Wedemeyer and Simmons[1] it was observed that normal mice placed in a hypoxia chamber shortly after or shortly before injection with an antigen responded to the hypoxia by extreme erythropoietic hyperplasia. They failed, however, to initiate an immune response in the usual manner seen in nonhypoxic controls. This finding suggested that a lethally irradiated animal under hypoxia might show a delay in developing the immunologic proliferation of the graft vs host reaction after foreign marrow transplant. Presumably the hemopoietic components of the transplant would proceed normally, but the immunologic component might experience sufficient retardation to make the response ineffectual.

Results and Conclusions. – A preliminary experiment in which mice were given 950 r of x irradiation and 40×10^6 isologous bone marrow cells and were placed in hypoxia chambers at 5 to 6% oxygen[2] for two weeks showed that the experiment could be successfully carried out under these circumstances. A few mice developed central nervous system symptoms and lesions[3] on removal from the chamber, but the final 90-day cumulative mortality in 32 animals was only 12.5%. It was also noted that hypoxia had relatively little effect on the time of death of x-ray control mice.

In the critical experiment 64 $1C3F_1$ male and female mice were given 950 r and 40×10^6 $B6D2F_1$ bone marrow cells. Four replicates with 16 mice each were run in this experiment. Half of each replicate was exposed to hypoxia for two weeks immediately following irradiation and half was kept in regular mouse cages.

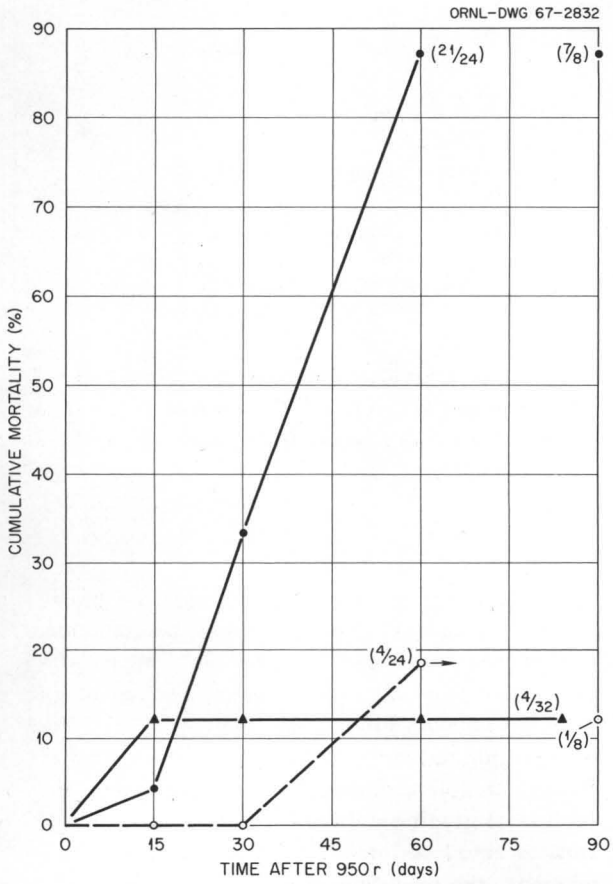

Fig. 11.1.1. Cumulative Mortality in Hypoxic and Nonhypoxic X-Irradiated Mice Given Homologous Bone Marrow. Symbols: 950 r plus 40×10^6 homologous bone marrow cells, ●——●; 950 r plus 40×10^6 homologous bone marrow cells and two weeks hypoxia, O---O; 950 r plus 40×10^6 isologous bone marrow cells and two weeks hypoxia, ▲——▲.

Figure 11.1.1 shows the expected high incidence of death from secondary disease under regular caging conditions but unusually low mortality of the hypoxic mice. Some of the replicate runs are not yet at 90 days after irradiation. Tests for chimerism of six 90-day survivors in the hypoxia groups and one 90-day survivor in the nonhypoxia mice showed 100% red blood cells of the donor type.

These findings support the idea of a priority in transplantation of hemopoietic and lymphatic tissues in the irradiated host with hemopoiesis taking precedence over a graft vs host reaction. In this context Kretchmar has pointed out the importance of a competition parameter and an environmental component which together determine the so-called "carrying capacity" of the host in analog computer models of the erythropoietic system.[4]

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11.2 SERINE-3-^{14}C METABOLISM IN IRRADIATION CHIMERAS: RESPIRATION PATTERN ANALYSIS

A. L. Kretchmar[1] C. E. Phipps[2]

Introduction. – We have reported previously[3] a study of serine-3-^{14}C metabolism in normal mice utilizing the experimental technique of respiration-pattern analysis. To study metabolism of a compound with this technique, one follows specific activity of expired CO_2 after injection of the compound labeled with ^{14}C. We found that by simultaneously injecting unlabeled compounds that were metabolically closely related to serine, information about certain specific reactions could be obtained from in vivo experiments.

Rate of appearance in respiratory CO_2 of an animal of ^{14}C from injected serine will clearly

depend upon many factors, among which are the relative activities of all enzymes of these and other pathways for serine, glycine, and formate metabolism; pool sizes of each of the intermediates; and the free serine and glycine concentration in liver, plasma, and muscle. Because of this complexity, respiration pattern analysis cannot be expected to give very detailed and quantitative information. The technique can, however, give useful qualitative and quite suggestive information, and it has the advantage of being a nondestructive analytical tool that can be applied with relatively minor disturbance to the intact organism.

Methods. – The techniques have been described.[3] Three groups of mice (eight to ten animals in each group) were studied: normal unirradiated mice and lethally irradiated animals (950 r total-body x irradiation) given either isologous bone marrow (IBM) cells (20×10^6) or homologous (HBM) cells (20×10^6).

Results and Discussion. – Simultaneous injection of unlabeled compounds with labeled serine influences respiration pattern in a specific way. Results are consistent with the following interpretation. When glycine is injected with the labeled serine, N^{10}-hydroxymethyl-THFA tends to react with glycine, thus inhibiting the appearance in the formate pool of ^{14}C from serine-3-^{14}C. With added glycine present, specific activity of respiratory CO_2 is lower than control for 45 min. On the other hand, when ammonium formate is injected with labeled serine, THFA can react with serine and then the N^{10}-formyl-THFA pool becomes more highly labeled. Moreover, ^{14}C in this pool would tend to become "trapped" in an expanded tissue formate pool and would be oxidized rapidly to $^{14}CO_2$. When formate was injected with labeled serine, specific activity of respiratory CO_2 was greater for 60 min than when labeled serine alone was injected. The bicarbonate pool of the animal is, of course, much larger than the formate pool so that addition of cold CO_2 from injected formate is relatively less important than the "trapping effect" of the increase in tissue formate.

Figure 11.2.1 shows that serine metabolism in irradiated mice treated with HBM is altered (21 days after treatment). Similarly irradiated mice given IBM have a nearly normal respiration pattern. The greater specific activity of respiratory CO_2 of mice treated with HBM could be due to

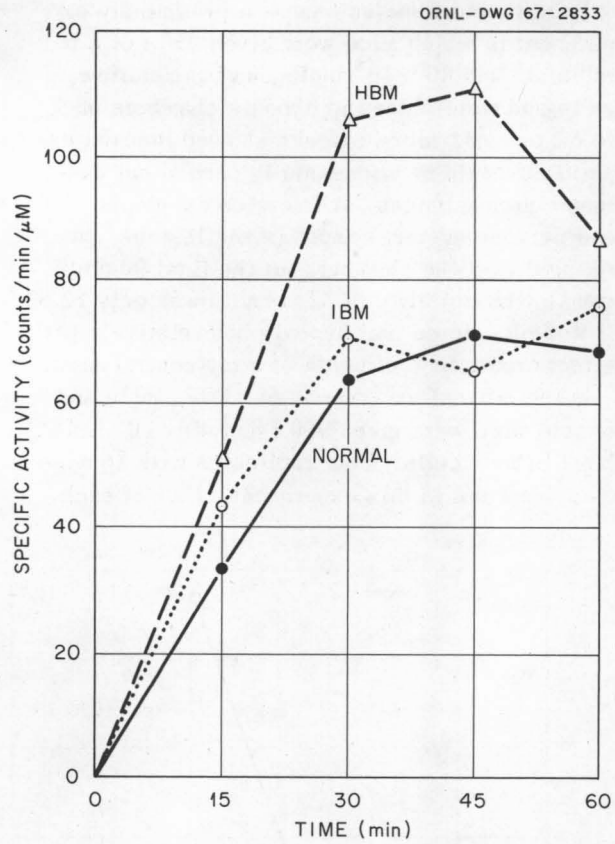

Fig. 11.2.1. The Specific Activity of Respiratory CO_2 After Injection of Serine-3-^{14}C in Normal Mice and in Lethally Irradiated Animals Given Isologous or Homologous Bone Marrow Cells.

less dilution of injected serine since we have previously shown[4] that at 21 days the concentration of free serine of liver tissue of chimeras is only about one-half that of normal mice or of irradiated animals given IBM. However, the effect of injecting unlabeled glycine or formate is different. In normal animals the simultaneous injection of glycine delays the appearance of ^{14}C from injected serine-3-^{14}C in respiratory CO_2; whereas, the injection of formate increases ^{14}C in respiratory CO_2. The results are similar in lethally irradiated mice treated with IBM. In irradiated mice given HBM, however, the effect of glycine is several times greater and the effect of formate is reversed. Thus, in chimeras, both glycine and formate decrease the rate of appearance of ^{14}C from serine into respiratory CO_2. This

suggests the possibility that in chimeras the metabolic system of Fig. 11.2.1 is somehow set to favor the production of serine and THFA.

To understand the biochemical details of this change in the metabolism of serine in chimeras will require experiments of a different kind than reported here, but respiration pattern analysis provides a useful tool for the survey of compounds whose metabolism might be altered in chimeras, and the results suggest specific reactions for further study.

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[1]Memorial Research Center, University of Tennessee, Knoxville, Tennessee; work carried out in collaboration with the Mammalian Recovery section.

[2]Medical Division, ORAU.

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11.3 PARASITES IN THE ALIMENTARY TRACT OF RADIATION CHIMERAS

W. H. McArthur[1]

Introduction. – Previous work from this laboratory regarding metabolism studies has suggested that homologous-bone-marrow-treated (HBM) animals, remaining in positive nitrogen balance, lose weight. Weight loss and nitrogen retention suggests storage in some abnormal compartment or compartments in animals with secondary disease.[2] This report is a continuation of previous studies on intestinal flagellates of mice with additional quantitative results in attempts to identify abnormal nitrogen storage.

Methods. – A total of 182 $(101 \times C_3H)F_1$/cum male or female mice 12 to 16 weeks old were used as follows: 52 normal, 24 x ray, 53 isologous bone marrow (IBM), and 53 HBM-treated animals. Mice were killed by decapitation on days 1, 4, 7, 10, 13, 14, 16, 20, 21, 25, and 30. Quantitative measurements of intestinal protozoa were made (flagellates per milligram of feces), smears were examined, and later, permanent slides were stained by a method previously reported.[3] The following flagellate parasites were encountered and identified in this study: *Giardia muris, Trichomonas muris,* and *Hexamita muris. Endameba muris,* a rhizopod, was found frequently.

Results and Conclusions. – In approximately 30% of the normal mice flagellates were not detected. When flagellates were found their numbers varied from about 10 to 10^4 in different mice. X-ray control animals showed a similar variation except that only 10% were flagellate free and several mice showed flagellate numbers in the millions. Compared with normal mice, IBM- and HBM-treated animals usually contained more flagellates, and a smaller percentage of the mice were flagellate free. In both marrow-treated groups flagellate numbers were variable from mouse to mouse (i.e., 10 to 10^8), and a clear trend of numbers with time after treatment is not apparent. Comparison of the two marrow-treated groups does not reveal a significant difference in the numbers of these intestinal protozoa. The data thus far indicate that intestinal flagellates probably contribute little to the nitrogen retention associated with secondary disease. Continued efforts are being made, however, to improve the quantitative and qualitative aspects of the method.

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11.4 ERYTHROPOIESIS IN THE MOUSE

L. H. Smith T. W. McKinley, Jr.

Introduction. – For several years we have used ^{59}Fe uptake by the spleen as a measure of proliferation of transplanted hemopoietic tissue to irradiated mice.[1] This end point is used to evaluate radiation effects on cells in terms of their reproductive capacity. Factors responsible for variation in results obtained by this method are not well defined; in fact, little is known about kinetics of iron uptake relative to age, sex, and

type of mouse. Therefore, a series of experiments was planned in an attempt to examine these variables, specifically in regard to how they might affect iron uptake values as measures of erythropoiesis. Results of the first part of this series are herein reported and describe the effects of age and sex on ^{59}Fe uptake in spleens and circulating erythrocytes of normal mice.

Materials and Methods. – (C3H × C57BL)F_1 mice were studied. Twenty-four-hour ^{59}Fe uptake in spleen and erythrocytes was measured[2] in males and females which were 7, 13, or 20 weeks old.

Results. – Figure 11.4.1 shows the percentage of injected ^{59}Fe appearing in the spleen and erythrocytes as a function of mouse age. The 20-week-old males and females showed a slight but significant ($P = 0.05$) increase in erythrocyte ^{59}Fe over the 7- and 13-week-old animals. On the other hand, spleen ^{59}Fe uptake was essentially constant with age, although uptake was consistently higher ($P < 0.05$) in females than in males.

Discussion and Conclusions. – In general these results show that in normal (C3H × C57BL)F_1 mice between 7 and 20 weeks of age, 24-hr uptake by spleen and erythrocytes is not grossly altered. Whether or not the slight increase in erythrocyte iron at 20 weeks is the beginning of a trend will only become apparent when values for 30- and 40-week-old mice become available. In addition, increase in erythrocyte iron may only reflect a decrease in blood volume with age rather than an increase in erythropoiesis. The sex difference in spleen iron uptake (female ~1.6 times male) is not due to differences in spleen weight. On the contrary, average spleen weight for males is slightly higher than that for females, and therefore, the erythropoiesis in the female spleen may be greater than that for the male of this hybrid. For both sexes there is also a slight rise in spleen weight with age which, in the face of constant iron uptake, would suggest a decrease in specific activity (counts min^{-1} mg^{-1}) of ^{59}Fe uptake with age.

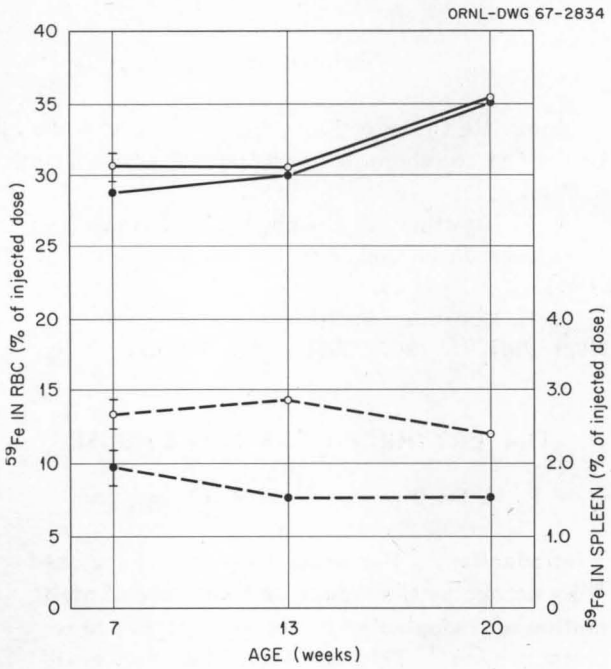

Fig. 11.4.1. Twenty-Four-hour ^{59}Fe in RBC and Spleen as a Function of Mouse Age. Vertical bars are representative standard errors. ● — ●, RBC ♂; ○ — ○, RBC ♀; ● --- ●, spleen ♂; ○ --- ○, spleen ♀.

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11.5 FURTHER STUDIES ON MODIFICATION OF POOR GROWTH OF PARENTAL MARROW CELLS IN F_1 HYBRID MICE

Joan Wright Goodman H. Brownell Wheeler[1]

Introduction. – In parent-hybrid combinations in which the poor-growth phenomenon has been observed, pretreatment of F_1 hybrid mice with parental spleen, either in the form of slice grafts placed on cut kidney surface or of dissociated cells administered intraperitoneally, results in good growth of subsequently transplanted parental bone marrow.[2] Pretreatment with suspensions of parental bone marrow, thymus, liver, lymph nodes, and spleens showed that only viable, immunologically competent cells produced a large augmentation in marrow growth. The present data are the result of investigation of two pretreatment parameters: (1) interval between pretreatment and irradiation (followed by marrow transplantation), and (2) pretreatment cell dose.

Materials and Methods.–Inbred parental lines were C57BL/Cum (called B) and C57BL/6 Jax

(B6), and their hybrid offspring: (B ♀ × C3 Anf Cum ♂)F_1 or BC3F_1, (B6 ♀ × DBA/2 ♂)F_1 Jax or B6D2F_1, and (B6 ♀ × A ♂)F_1 Jax or B6AF_1. Hybrids were exposed to 900 r whole-body x irradiation and 18 to 22 hr later were given 10^6 parental bone marrow cells intravenously. Seven days after marrow transplantation proliferation of erythropoietic elements was assessed by measuring 24-hr ^{59}Fe incorporation into erythrocytes and spleens of chimeras.

Pretreatment consisted of an intraperitoneal injection of a given number of eosin-negative parental spleen cells at a specified period of time (e.g., ten days) before irradiation.

Results and Conclusions. – Because early experiments had suggested that modification of poor growth varied with time of pretreatment, three experiments were carried out to explore this variable. The results are shown in Fig. 11.5.1 with a standard pretreatment spleen cell dose of 3×10^7. It can be seen that this number of cells has a maximum effect when administered two to three weeks before marrow transplantation. This augmentation decreases after three to four weeks, but the actual course of the decline is obscured by experimental variability. The fact that time is required for the modification to take place could mean that the spleen cells must proliferate in order to be effective. If this is the case, then one should be able to obtain the same degree of augmentation by shortening the interval between pretreatment and irradiation and administering a greater spleen cell dose.

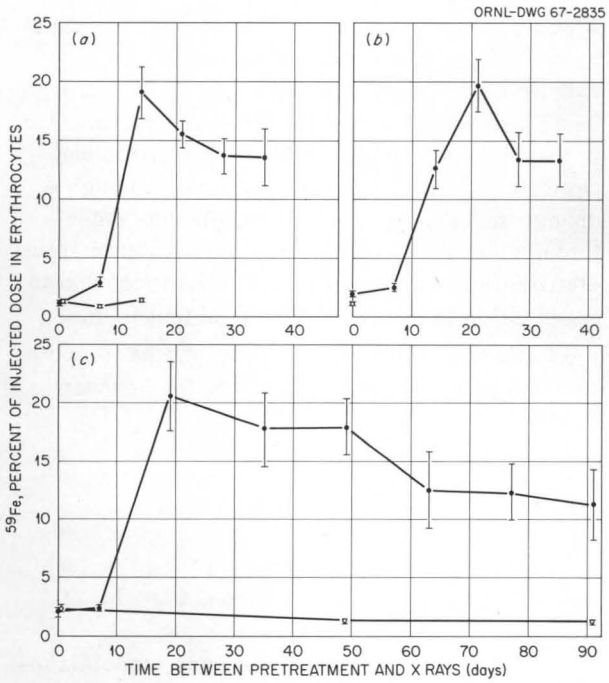

Fig. 11.5.1. ^{59}Fe Uptake in Pretreated P → F_1 Chimeras. Recipients in *a* and *c* B6D2F_1, in *b* BC3F. 3×10^7 eosin-negative B6 or B spleen cells given at preirradiation times indicated.

Two experiments in which the pretreatment-irradiation interval was constant and spleen cell dose was varied are shown in Table 11.5.1. In the first experiment B6 cell doses varying from 10 to 160 million cells were administered ten days prior to irradiation. Subsequent erythropoiesis generally increased with increasing spleen cell dose, although differences from one group to the next were not significant at the 5% level. In the second experiment lower doses of these B6 spleen cells, varying from 3×10^4 to 10^7 cells, were administered 16 days prior to irradiation. All doses tested showed some increase over the control group, but at the low dose ranges this increase was so slight as to be inconclusive. Again, there tended to be an increased response noted in the higher dose ranges. Although a more thorough exploration of these possibilities in repeated experiments might reveal a clear-cut pretreatment dose effect, the experiment is limited on the one hand by the difficulty in supplying cell numbers that might be comparable with the progeny of any given inoculum after a logarithmic growth phase and on the other, by physiologic factors that might limit the persistence and growth of parental spleen cells in the hybrid host.

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[1]Veterans Administration Hospital, West Roxbury, Mass.

[2]J. W. Goodman and H. B. Wheeler, *Nature* **211**, 1420 (1966).

11.6 A FACILITY DESIGNED FOR PATHOGEN-FREE INBRED MOUSE PRODUCTION

M. L. Simmons L. P. Wynns[1]
E. E. Choat[1]

During the past three years a new building has been designed and constructed at the Biology Di-

Table 11.5.1. Effect of Pretreatment Cell Dose on Modification of Poor Growth

Irradiated Recipient	Pretreatment[a] Dose of Spleen Cells (Millions)		Numbers of Mice	^{59}Fe Uptake by[b] Erythrocytes	Spleen
B6D2F$_1$	10		26	8.4 ± 1.1	6.3 ± 0.6
	20		21	4.9 ± 0.8	3.7 ± 0.6
	40	B6	25	8.3 ± 1.1	9.1 ± 0.9
	80		19	11.1 ± 1.2	11.7 ± 0.9
	160		24	13.0 ± 1.7	10.7 ± 1.0
	160	B6D2F$_1$	24	1.0 ± 0.1	0.4 ± 0.0
B6AF$_1$	0.03		31	2.13 ± 0.2	0.86 ± 0.2
	0.3		30	1.74 ± 0.1	0.59 ± 0.0
	1	B6	28	2.45 ± 0.3	1.13 ± 0.2
	3		30	2.71 ± 0.4	1.78 ± 0.3
	10		22	7.89 ± 1.5	4.88 ± 0.8
	10	B6AF$_1$	32	1.16 ± 0.1	0.38 ± 0.0

[a]All spleen cells given intraperitoneally 10 days in first and 16 days in second experiment before x rays and 10^6 B6 bone marrow.

[b]Means ± S. E.

vision. It was designed to produce pathogen-free animals or to receive pathogen-free animals from commercial sources for use in the 6th Floor Experimental Animal Facility and Carcinogenesis programs. The building is now completed and will be utilized as a mouse production facility for the production of inbred mice.

The concept of using animals with a known or defined flora – "gnotobiotes" – is not a new one. It has been studied in this laboratory extensively by Upton and collaborators. However, the concept of utilizing a "barrier" type animal facility capable of being used for pathogen-free animals in combination with a filter-top cage system[2] is quite recent.[3] We believe that by defining "specific-pathogen-free" animals as animals free of known pathogenic viruses, bacteria, and parasites we can utilize the combination facility and filter-top system to achieve an SPF-type animal production. It is anticipated that the filter-top caging, so effective in preventing infection from occuring in a conventional environment,[4,5] will be a major factor as a final line of defense within a barrier system.

The facility was built for the specific purpose of controlling the environment such that a minimal number of infectious organisms enter the area in which these animals live. This is accomplished by controlling the movement and condition of air, supplies, personnel, and animals. There are five basic methods of entry into the barrier area. One is for personnel, and four are for materials and equipment. Personnel may enter only through a change and shower area. Materials may enter through chemical sterilization, high-vacuum steam sterilization, ethylene oxide sterilization, and the combination cage and bottle washing machine.

We anticipate that this facility will be in operation and producing animals during the next few months.

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11.7 DISTRIBUTION OF ANTIBODY TO VIRUSES IN EXPERIMENTAL MICE

R. W. Tennant Roberta K. Reynolds
K. R. Layman

Introduction. – The consequences of epizootic virus diseases in experimental mice are well documented, particularly in the cases of ectromelia and epizootic diarrhea of infant mice. Less well known are the effects of enzootic infections in experimental animals, primarily since evaluation of the loss of an experiment often requires additional investigative effort. Indicative of the magnitude of this problem are the recent findings that 69 of 71 experimental tumors harbor at least one extraneous murine virus.[1]

Methods. – Sera from mice were tested against the various viruses by hemagglutination inhibition or complement fixation with techniques previously described.[2] All tests were performed in the Microtiter system, and positive and negative control sera and antigen and complement titrations were included with each test series. Tests were rejected when these controls varied more than twofold in dilution or greater than two CH_{50} complement units.

Conclusion. – The testing program, in effect since November 1966, has yielded the following preliminary conclusions based upon the data presented in Table 11.7.1: (*a*) Virus infections which are not present in mice from the major suppliers are perpetuated in experimental animal facilities. (*b*) Conversely, germfree mice and mice reared under barrier conditions in the Biology Division are free from antibody to the test viruses. (*c*) Mice derived from germfree stock but reared under conventional conditions in filter-top cages are spared common virus infections.

References

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Table 11.7.1. Antibody Status by Virus[a]

Test Animal		Reovirus Type 3	Pneumonia Virus of Mice (PVM)	Encephalomyelitis (GDVII)	Polyoma (Parotid Tumor) Virus	Newborn Pneumonitis (K Virus)	Sendai (Pneumonitis)	Mouse Adenovirus	Mouse Hepatitis (Polyvalent Antigen)	Rat Virus (Kilham)
				Outside Sources						
Balbc/Cum		0/25	0/25	18/25	0/25	0/25	0/25	0/16	1/8	
	%+			72					12	
C3H/Anf/Cum		0/22	0/23	18/18	0/20	0/24	0/25	0/22	0/7	
	%+			100						
DBA_2/JAX		21/25	3/25	0/25	0/25	0/25	0/25	0/25	5/25	
	%+	84	12						20	
Rats		0/35	16/36	0/36	0/36	0/36	15/36	0/36	8/36	21/36
	%+		44				47		31	59
				Internal Sources						
BC_3F_1/C		0/15	16/17	14/17	0/17	0/17	16/17	0/11	0/15	
	%+		94	82			94			
RFM/9N (6th floor 9207)		1/22	4/21	19/25	6/25	0/29	18/25	0/19	2/6	
	%+	5	19	76	24		72		33	
$B_6D_2F_1$		14/28	26/26	19/30	1/29	0/28	26/26	0/30	20/30	
	%+	50	100	63	3		100		66	
Germfree (9207)		0/20	0/25	0/25	0/24	0/25	0/21	0/21	0/20	
Barrier (9211), $C57B_1$/6		0/64	0/70	0/68	0/68	0/67	0/68	0/66	0/60	
Conventional filter top, $C57B_1$/6 (9769)		0/50	0/5	0/50	0/50	0/50	0/50	0/50	0/50	

[a]Number positive/total tested.

11.8 THE ISOLATION OF *SALMONELLA POONA* AND A NONMOTILE VARIANT FROM LABORATORY MICE

J. A. Franklin C. B. Richter

Introduction. – Laboratory mice may carry salmonella as part of their intestinal flora without any overt effects. However, high mortality may occur in an epidemic situation. The *Salmonella* species most often encountered in laboratory mice are *S. typhimurium* and *S. enteritidis*.

Results and Discussion. – During the course of routine fecal sampling of mice arriving at the receiving and quarantine facility of this laboratory we have isolated and identified *Salmonella poona*, which heretofore has not been reported in laboratory mice. The organism was isolated from three inbred strains of mice, all of which came from the same commercial source. This organism produces the typical salmonella biochemical reactions, agglutinates in group G antisera, and possesses somatic antigens 13 and 22 and flagellar antigens *z*, 1, and 6. Prior to the isolation of this organism all salmonella isolates from this commercial supplier's mice had been *Salmonella typhimurium var. copenhagen*.

An interesting sidelight to the isolation of *S. poona* has developed in that some of the recent isolates have been nonmotile. These nonmotile Salmonella possess the same somatic antigens as *S. poona* but, being devoid of flagella, cannot be specifically typed. Rakieten[1] and Rakieten and Bornstein[2] reported the isolation of a bacteriophage from sewage which was capable of lysogenizing *S. poona*, a process which caused concomitant loss of motility and H antigens.

We have performed the conventional techniques (chloroform extraction and ultraviolet irradiation) with the *S. poona* and the nonmotile salmonella cultures isolated. Both methods have failed to demonstrate the presence of phage or prophage. In addition, repeated subculturing of both organisms to a variety of media has not resulted in any change in their motility. This suggests that the character of this particular salmonella is not due to phage action.

All specific typing of isolates was conducted through the courtesy of Dr. W. H. Ewing, Enteric Bacteriology Unit, Communicable Disease Center, Atlanta, Georgia.

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Pathology and Physiology

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12.1 PRELIMINARY DATA ON THE LONGEVITY AND LEUKEMIA INCIDENCE IN MICE OF THE BARRIER-SUSTAINED RFM/UnF SUBLINE TO BE USED IN THE LOW(MEDIUM) LEVEL EXPERIMENT

All Members of Pathology-Physiology Section

Introduction. – RFM/UnF mice derived from germfree ancestors and specifically contaminated with a defined, nonpathogenic microflora are being used to investigate the long-term somatic effects of low-level whole-body gamma irradiation.[1] To compare the response of these mice with that of their conventionally reared counterparts, a pilot experiment is being carried out on the animals preparatory to the main experiment. Preliminary results of the pilot study, concerning the longevity and incidence of leukemia in the mice of the new subline, are summarized in the following.

Results and Discussion. – Data from animals followed until 20 months of age imply that mice of the barrier-sustained subline have a higher incidence of radiation-induced lymphomas and a lower incidence of granulocytic leukemias than their conventional counterparts (Fig. 12.1.1). Comparable differences in longevity are not evident, although the results suggest that males of the new subline may outlive their conventionally reared counterparts; that is, more than 60% of the nonirradiated barrier-sustained males have survived for 700 days, whereas the mean survival time of conventionally reared nonirradiated males is 595 days.

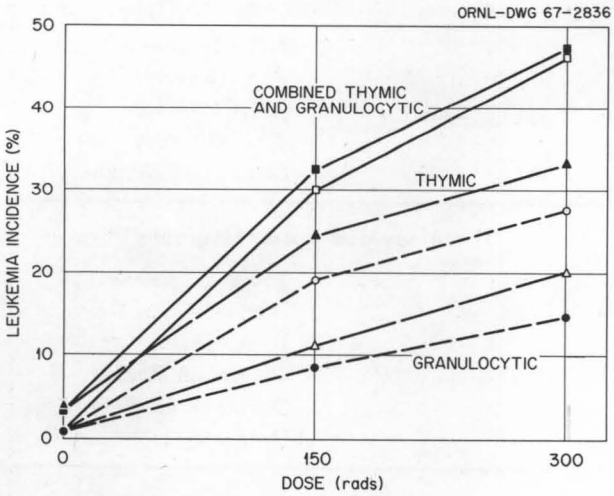

Fig. 12.1.1. Incidence of Radiation-Induced Leukemias in Barrier-Sustained RFM/UnF Male Mice, as Compared with That in Their Conventionally Reared Counterparts. Shaded symbols, barrier-sustained mice; nonshaded symbols, conventional mice. O = granulocytic leukemia, Δ = thymic lymphoma, □ = combined neoplasms.

The lower incidence of myeloid leukemia in the mice of the new subline is consistent with results in germfree mice and mice of another barrier-sustained colony.[2] It may tentatively be attributed, at least in part, to the pathogen-free nature of the microflora in the barrier-sustained mice. It is noteworthy that the lower incidence of granulocytic leukemia is paralleled by a higher incidence of lymphoid leukemia, with the result that the combined incidence of both neoplasms in the new subline is similar to that in the old.

Although the results to date are preliminary, they give no indication that the barrier-sustained subline will be unsatisfactory for the Low (Medium) Level Experiment. They do, however, indicate differences in radiation response between the new subline and the old, which must be considered in the final design of sample sizes for the experiment.

References

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12.2 POLYPLOIDY AND MATURATION OF MEGAKARYOCYTES

T. T. Odell, Jr. C. W. Jackson
Rebecca S. Reiter

Introduction. – The maturation of megakaryocytes is still not well understood, although it is known that mature megakaryocytes are polyploid, some of them having ploidy values up to 32*N* (16 times the diploid amount of DNA). Based on earlier work, it has been postulated that megakaryocytes first go through a period of DNA replication to attain superdiploid values, and then their cytoplasm matures. These investigations were undertaken to study the relation between megakaryocyte morphology and DNA content.

Results and Discussion. – Megakaryocytes of rat bone marrow were classified into types I, IIa, and IIb, largely by the amount of granulation in their cytoplasm. Type I cells had no granulation,

Table 12.2.1. Distribution of Morphological Types of Megakaryocytes in Various Ploidy Classes

Morphological Type	Distribution of Megakaryocytes in Ploidy Classes[a]								All Ploidy Classes,[a] 4N--32N	
	4N[b]		8N		16N		32N			
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
I	6	100	32	48	14	11	2	14	54	25
IIa	0	0	13	19	25	19	1	7	39	18
IIb	0	0	22	33	90	70	11	79	123	57

[a]The values shown are based on a total of 216 megakaryocytes analyzed in one rat.

[b]2N = diploid chromosome complement (no recognizable megakaryocytes were encountered with 2N ploidy).

type IIa cells a small amount, and type IIb a moderate to large amount. The amount of DNA in individual nuclei was determined by microspectrophotometry (Table 12.2.1). Type I cells had ploidy values of 4N to 32N (no type I cells were encountered with 2N ploidy). The majority of type IIa and IIb cells had DNA values in the 16N or 32N range, but some were 8N. The finding that only type I cells were initially labeled after a single injection of tritiated thymidine and that the label was progressively seen in cells of the remaining types indicated a progression of labeled megakaryocytes through these morphological compartments.

From these results it appears that a megakaryocyte must have a DNA value of at least 8N before its cytoplasm can differentiate beyond the morphological type I stage; the majority of cells with maturing cytoplasm have ploidy values of 16N or 32N. These results confirm the prediction that megakaryocytes with various ploidy values may become cytoplasmically mature. They also imply that platelets may be derived from megakaryocytes of different ploidy values.

12.3 ESTIMATION OF THE FREQUENCY DISTRIBUTION OF MEGAKARYOCYTES IN PLOIDY CLASSES

D. G. Gosslee[1] T. T. Odell, Jr.
C. W. Jackson

Introduction. – A procedure for estimating the proportion of megakaryocytes in each of several stages of nuclear replication (ploidy) was previously reported.[2,3] Recent measurements of the DNA content in each of 3000 cells from nine male rats were analyzed in a similar manner. In addition, two other statistical methods of estimation were used. The three methods were compared by their performance on the experimental data and particularly on numbers generated to simulate the data.

Results and Discussion. – The three methods of estimation are: (1) least squares with the weight for each interval equal to the expected frequency of cells in the interval, the method previously reported; (2) least squares with the weight equal to the reciprocal of the variance of the expected frequency; and (3) maximum likelihood. Since difficulties in obtaining convergence are not uncommon with nonlinear least-squares computing programs having a large number of parameters, assumptions of equal variances and known distances between means were made for methods 1 and 2 in order to reduce the number of parameters. Since the computing procedure for method 3 is faster and more direct, and thus feasible for more parameters, the assumptions were relaxed for this method.

Random normal deviates, which have a mean of zero and a variance of 1, $N(0, 1)$, were transformed into normal populations, $N_i(M_i, \sigma^2)$, $i = 1, 2, 3$, with frequencies chosen to simulate the megakaryocyte data (see "target percentages" in Table 12.3.1). In addition, the variance was changed to obtain five sets of three populations each with varying degrees of overlap. Each method was applied to the simulated data to demonstrate its accuracy and efficiency in estimating the known proportions (Table 12.3.1).

As would be expected, all three methods yielded excellent estimates when the variance was small.

Table 12.3.1. A Comparison of Three Methods of Estimating Frequencies in a Mixture of Three Distributions

The test data used were generated to simulate the observed distribution of megakaryocytes in three ploidy classes

σ, Standard Deviation[a]	Method[b]	Percentage of Population in Each Ploidy Class		
		8*N*	16*N*	32*N*
	Target[a]			
		9.275	71.304	19.420
	Calculated			
0.0418	1	9	71	20
	2	9	71	20
	3	9	71	19
0.05225	1	8	71	21
	2	10	71	19
	3	10	71	19
0.0627	1	8	74	18
	2	9	72	19
	3	10	71	19
0.07315	1	9	72	18
	2	10	71	19
	3	10	70	20

[a]The target percentages and standard deviations (σ) were chosen to be typical of values observed in data on rat megakaryocytes.

[b]The three methods are described briefly in the text. Number of "cells" = 345.

As the variance increased, method 1 became less accurate than the other two methods. Methods 2 and 3 were demonstrated to be accurate within the range of overlap in the experimental data. Method 3 is preferred since it can be applied without restrictions on the means and variances. All three methods have approximately the same efficiency as judged by the standard errors of the estimates of the proportions. The means and variances were estimated well by all three methods. The estimates of percentages (proportions) in corresponding stages between the two sets of megakaryocyte data differed by 1% (0.01) or less for method 3 but by a larger amount for methods 1 and 2.

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12.4 INFLUENCE OF THE MICROBIAL ENVIRONMENT ON THE DEVELOPMENT OF MYELOID LEUKEMIA IN X-IRRADIATED RFM MICE

H. E. Walburg, Jr. G. E. Cosgrove

Introduction. – Mice of the RF strain have demonstrated a markedly increased incidence of myeloid leukemia following exposure to ionizing radiation.[1] To determine what role, if any, the microflora plays in the pathogenesis of myeloid leukemia, the incidence of the disease has been studied in irradiated sublines maintained in different microbial environments.

Results and Discussion. – Five- to six-week-old male and female RFM mice were exposed to 300 r of x radiation and examined throughout their life-span for mortality and incidence of diseases. Only one case of myeloid leukemia was observed in germfree mice (Table 12.4.1), giving an incidence of 1% in the germfree males, whereas the incidence in their conventionally reared counterparts was 13%. A similar difference in incidence occurred between germfree and conventional females. Males subjected to microbial shock by transfer from an environment containing a limited number of bacteria to a conventional microbial environment showed the highest incidence (namely 25%), approximately twice that seen in males exposed to the conventional microbial environment

Table 12.4.1. Incidence of Myeloid Leukemia in Irradiated RFM Mice Reared in Different Microbial Environments

Sex	Germfree	Conventional	Barrier to Conventional
Male	1/79 (1.3%)	12/94 (12.8%)	22/86 (25.6%)
Female	0/91 (0.0%)	7/85 (8.2%)	8/123 (6.5%)

throughout life. Genetic differences were minimal since all mice were less than five generations removed from a common inbred ancestor.

These results suggest that the incidence of myeloid leukemia varies inversely in relation to the bacterial "cleanliness" of the environment, possibly as a result of associated variations in the rate of granulocytopoiesis. Such an interpretation, if true, would help to explain the reduced incidence of myeloid leukemia that has been reported[2] in x-irradiated conventionally reared mice over the past several years, during which many improvements in husbandry and sanitation have been instituted in our animal colonies.

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12.5 METHYLCHOLANTHRENE-INDUCED MYELOID LEUKEMIA IN GERMFREE RFM MICE

H. E. Walburg, Jr. G. E. Cosgrove

Introduction. – Myeloid and lymphoid leukemia have been produced in conventional mice of the RF strain by oral administration of 20-methylcholanthrene, and the latent period of such leukemias has been reduced by painting the mice with a croton oil–benzene solution.[1] This experiment was undertaken to determine whether similar treatment would induce myeloid leukemia in germfree RF mice, which are relatively resistant to the induction of the disease by radiation.

Results and Discussion. – Five- to six-week-old germfree RFM mice, which show a greatly reduced incidence of radiation-induced myeloid leukemia when compared with their conventionally reared counterparts, were treated orally three times a week with 0.1 ml of an 0.8% solution of 20-methylcholanthrene in olive oil. In addition, half the mice were painted on the abdomen twice a week with a 5% solution of croton oil in benzene. All groups of mice which received methylcholanthrene had an incidence of leukemia approaching 100% (Table 12.5.1). In all but one group these leukemias were exclusively of the lymphoid type. In male RFM mice which received both methylcholanthrene and croton oil–benzene, four cases of myeloid leukemia were observed. This incidence, 10.5%, is approximately that seen in irradiated conventional males of the same strain (13%) and is considerably higher than that seen in irradiated germfree males (1%).

Irino and coworkers[1] saw an increase in peripheral granulocytes following painting with croton oil in benzene, suggesting an increase in granulocytopoiesis. The increase in incidence of methylcholanthrene-induced myeloid leukemia in germfree RFM males painted with croton oil in benzene may, therefore, be due to increased granulocytopoiesis.

Table 12.5.1. Incidence of Methylcholanthrene-Induced Leukemia in Germfree RFM Mice

Sex	Treatment	Myeloid Leukemia	Lymphoid Leukemia	All Leukemia
Female	Methylcholanthrene only	0/27 (0.0%)	25/27 (92.6%)	25/27 (92.6%)
Female	Methylcholanthrene + croton oil in benzene	0/30 (0.0%)	28/30 (93.3%)	28/30 (93.3%)
Male	Methylcholanthrene only	0/21 (0.0%)	20/21 (95.2%)	20/21 (95.2%)
Male	Methylcholanthrene + croton oil in benzene	4/38 (10.5%)	34/38 (89.5%)	38/38 (100.0%)
Female	Croton oil in benzene only	0/11 (0.0%)	0/11 (0.0%)	0/11 (0.0%)
Male	Croton oil in benzene only	0/11 (0.0%)	0/11 (0.0%)	0/11 (0.0%)
Female	No treatment	0/10 (0.0%)	0/10 (0.0%)	0/10 (0.0%)
Male	No treatment	0/7 (0.0%)	0/7 (0.0%)	0/7 (0.0%)

References

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12.6 SOME CHARACTERISTICS OF TRANSPLANTABLE MYXOFIBROSARCOMAS

R. L. Tyndall Ernestine Teeter
R. C. Brown J. A. Otten

Introduction. – It was previously reported that an established culture of BALB/c mouse spleen and thymus cells (JLS V6) can be transformed morphologically by the Rauscher leukemia virus. This transformation is characterized by loss of contact inhibition and marked production of mucopolysaccharide (MPS). Such cells injected into newborn BALB/c mice form myxofibrosarcomas at the site of inoculation. Uninfected cells, on the contrary, show no such MPS production and are less tumorigenic on injection into newborn mice, in which they give rise to spindle cell sarcomas instead of myxofibrosarcomas.[1] This study was carried out to characterize further the transformation induced by the Rauscher virus.

Results and Discussion. – To study the MPS associated with the myxofibrosarcomas, unstained sections of tumor were overlaid with bovine and/or bacterial hyaluronidase, trypsin, or sialidase for 18 hr at 37°C. Following this incubation, the sections were stained for MPS with colloidal iron. It was seen that tumors treated with either bovine or bacterial hyaluronidase were devoid of MPS, whereas those treated with trypsin or sialidase showed little or no effect on MPS content. These results indicate that the MPS in the tumor is composed primarily of hyaluronic acid derivatives.

To study the tumorigenicity of transplantable myxofibrosarcomas, 1×10^5 tumor cells were injected subcutaneously into one-, two-, three-, or four-day-old BALB/c mice. It was seen that tumors developed in all mice injected at one or two days after birth, whereas mice injected three days after birth were only partially susceptible to tumor induction, and mice injected four days after birth were resistant. These results imply that the myxofibrosarcoma cells can be recognized as antigenic by an immunologically competent mouse, as has been suggested also by the observation that newborn mice are protected against challenge with these tumor cells if their mother has been immunized with Rauscher virus vaccine.[2]

Electron microscope studies, undertaken to determine if virus particles are present in the passaged myxofibrosarcomas, have revealed leukemia virus-like particles in the tumor and in spleen tissue from tumor-bearing animals. Studies are continuing in an effort to identify the observed virus particles and to explore their role in the antigenicity and MPS production of the myxofibrosarcoma cells.

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12.7 ALTERED ESTERASE ZYMOGRAMS ASSOCIATED WITH LEUKEMIA VIRUS INFECTION

R. L. Tyndall R. C. Allen
Dorothy J. Moore

Introduction. – Gross cytopathic effects (CPE) have been observed in an established culture of BALB/c mouse spleen and thymus cells following infection with the Rauscher leukemia virus. The infected cultures (JLS V5) also show a marked increase in mucopolysaccharide (MPS) production, with a concomitant loss of contact inhibition. The decreased contact inhibition results in a transformed, "crisscross" growth pattern. Such infected, transformed cells are highly tumorigenic when injected into newborn BALB/c mice. The resultant tumors are rich in MPS and are classified as myxofibrosarcomas.[1] The uninfected control cells (JLS V6) do not show CPE or transformation and are much less tumorigenic. Moreover, the tumors that do result from injection of noninfected control cells are classified as spindle-cell sarcomas.[1] The stimulation of MPS formation associated with the neoplastic transformation induced by the Rauscher virus prompted an investigation into the effect of the virus on protein and enzyme synthesis.

Results and Discussion. – Cell pellets of different sublines of control (JLS V6) and infected (JLS V5) cultures were homogenized with tissue grinders, and esterase zymograms of the resultant

extracts were prepared by disk electrophoresis as previously described.[2] Extracts from infected cells in each of three separate experiments showed a marked increase in esterase activity which was attributable to the appearance of "new" esterase bands (Fig. 12.7.1).

The significance of the increase in esterase activity and the presence of "new" esterase isozymes in relation to the altered MPS synthesis and oncogenicity of Rauscher-virus-infected cells requires more extensive investigation. A question of particular interest is whether the code for the "new" esterases is of cellular or viral origin. Moreover, it is of primary importance to determine whether the alteration of esterase patterns seen in the infected cells is a peculiarity of this particular cell-virus system or represents a general phenomenon associated with viral neoplastic transformation.

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12.8 THE USE OF GERMFREE MICE TO MONITOR THE MICROBIOLOGICAL ENVIRONMENT

R. C. Allen
R. T. Jones
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J. H. Chandler, Jr.
G. W. Fortner

Introduction. – In order to supplement routine bacteriological methods for determining the effectiveness of a barrier animal facility[1] in reducing the microbial content of the environment, five cages of germfree mice and two cages of monocontaminated mice were placed in a part of the facility previously decontaminated with β-propiolactone vapor.

Results and Discussion. – Within the first 24 hr after the cages were placed in the facility, *Staphylococcus albus* was found in feces from mice in one of the seven cages. By the end of seven days, an organism (or organisms) with fusiform morphology was found in fecal samples from mice in all cages, a *Corynebacterium* species was present in one cage, and *Streptococcus faecalis var. liquifaciens* was present in all but one cage.

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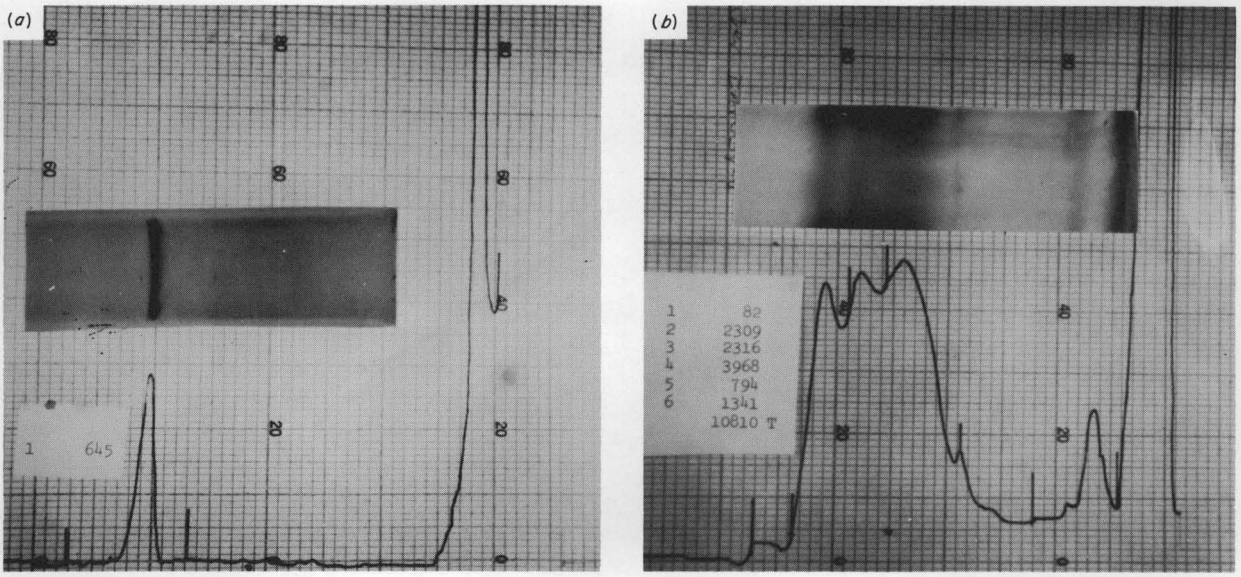

Fig. 12.7.1. Esterase Zymograms of Control and Rauscher-Virus-Infected Cell Cultures, with Corresponding Densitometric Recordings. (*a*) Control cells, 15.1 mg protein extract, showing only one enzyme band and corresponding densitometric recording. (*b*) Cells infected with Rauscher virus, 9.93 mg protein, showing seven resolvable bands of esterase activity and their corresponding densitometric recording. Figures to left of *a* and *b* are the integrated values of the area under each peak in the densitometric recording delineated by the vertical slash marks.

All of the above organisms except the fusiform bacteria were detected by means of surface swabbing, contact plates, and air-sampling. The fusiform bacteria could be detected only on direct examination of smears of feces from the mice and could not be cultured on ordinary aerobic or anaerobic media. However, it has since been found that at least three morphologic types of fusiform bacteria can be isolated from the mice by heat shock and subsequent culture on specialized media developed for fastidious anaerobic bacteria. The organisms are spore-forming bacteria thought presently to be types of clostridia.

The source of the fusiform bacteria and the enterococcus, both of which are indigenous to conventional mice, is under investigation. The ability of the germfree mouse to enable the detection of bacteria such as the fusiform organism, which would go undetected by conventional techniques, extends our ability to characterize the microbial composition of the environment.

References

[1] *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1964,* ORNL-3700, pp. 125–26.

Plant Physiology and Photosynthesis

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Plant Physiology (13.1–13.4)

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Photosynthesis (13.5)

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13.1 GROWTH PROMOTION AND INHIBITION RESULTING FROM REIRRADIATION OF GAMMA-PLANTLETS

A. H. Haber Pat C. Kerr
D. E. Foard

Introduction. – After receiving doses of 500 to 800 kilorads, wheat seedlings ("gamma-plantlets") can germinate and grow without undergoing cell divisions but otherwise show no detectable injury resulting from the seed irradiation. One possible test of the notion that except for the absence of cell division there is little other radiation injury might be expected to come from a study of the sensitivity of growing gamma-plantlets to seedling reirradiation. This report presents some results of such an investigation as well as some unexpected findings concerning a possible mechanism for radiation-induced growth stimulation.

Results and Discussion. – Gamma-plantlets from either 500- or 800-kilorad seed irradiation differed in growth only very slightly. When such gamma-plantlets were reirradiated as three- or four-day-old intact seedlings, that is, attached to the endosperm, there was a significant reduction of growth by only 50 kilorads. After reirradiation with 20 to 25 kilorads, however, there was an increased growth of both the first and second foliage leaves, but not of the coleoptile. When the growing gamma-plantlets were removed from their endosperm reserves just before reirradiation, their subsequent growth could be supported by a nutrient medium containing sucrose. Under these conditions, however, the seedlings removed from their endosperm showed no radiation-induced stimulation but rather a reduction of leaf growth after 25 kilorads. These results on promotion and reduction of gamma-plantlet growth by seedling reirradiation doses that are small relative to the seed irradiation doses given initially

emphasize the normal aspects of gamma-plantlet growth even with respect to irradiation itself. The requirement of the endosperm for the stimulation of leaf growth by 20- to 25-kilorad reirradiation suggests that this stimulation is mediated by an endosperm effect. Preliminary experiments indicate that the activity of α-amylase remaining in and released from the irradiated seeds is stimulated by reirradiation with 25 kilorads. It remains to be seen whether this increased enzyme activity results from greater de novo synthesis, greater release of preformed enzyme, or altered contents of activators or inhibitors of enzyme activity. Nevertheless, the increase in α-amylase activity, which could contribute to seedling growth only when the endosperm is present, may underlie the growth stimulation from 25 kilorads.

13.2 ACTIONS OF GAMMA RADIATION ON SENESCENCE AND DEATH IN EXCISED WHEAT LEAF TISSUE AFTER NORMAL CESSATION OF CELL GROWTH AND DIVISION

A. H. Haber W. D. Willis

Introduction. – Excised leaf culture is a system that has been of great interest and value in the study of senescence. This is especially true with excised wheat leaves. We showed previously that doses as high as 100 kilorads did not appreciably alter the time course of senescence in this tissue.[1] Presumably, the great radioresistance of this senescence can be attributed to the absence of the typical radiation effects of genic imbalance resulting from chromosome breakage in dividing cells, radiation-induced mitotic inhibition, and radiation-induced growth inhibition.

This report describes the effects of gamma radiation on the dark senescence of excised mature wheat leaf tips, using for an index of senescence the decay of chlorophyll, which reflects deterioration of chloroplasts. In darkness, moreover, no anabolism of chlorophyll occurs. The dark decay was also compared with decay in illuminated tissues. Senescence, which leads to death, is, nevertheless, distinct from death. To complement the findings of radiation sensitivity of senescence, this study also attempts to establish doses and biological criteria for radiation-induced death itself in this nongrowing, nondividing system.

Results and Discussion. – Wheat grains were germinated and grown in an illuminated (16-hr photoperiod) growth chamber. On the seventh day after sowing, the apical 2 cm of the first foliage leaf was harvested and floated on distilled water in 60-mm plastic petri plates. Cell elongation and cell division had already ceased in this tissue before the time of harvesting. After this time, the tips were stored, transferred, and gamma irradiated in darkness. After irradiation the leaf tips were floated on an aqueous 10^{-3} *M* potassium phosphate buffer at pH 6.0 or on 10^{-5} *M* kinetin in 10^{-3} *M* phosphate buffer at pH 6.0 in darkness in growth chambers with various constant temperatures between 10 and 40°C. The leaf tissue was collected at intervals and analyzed for chlorophyll content. It was possible to establish the occurrence of gamma-radiation-induced death by four criteria: (1) photodestruction of chlorophyll in the light, (2) inhibition of chlorophyll decay in darkness, (3) loss of chemical regulation of chlorophyll decay, and (4) abolition of a temperature optimum (at 30°C) for chlorophyll decay in darkness. By all four criteria, gamma-radiation-induced death occurs only after doses greater than 0.5 megarad. Doses up to 0.5 megarad did not appreciably affect any of the following dark decay characteristics: rate at any of the temperatures studied, temperature optimum, and sensitivity to kinetin. These results indicate that sufficiently massive doses, greater than 0.5 megarad, can kill this tissue. However, massive doses less than 0.5 megarad are neither lethal nor do they significantly promote senescence. This insensitivity of senescence to gamma radiation is in marked contrast to our findings, in these same experiments, concerning the ease with which senescence can be altered by chemicals or temperature. These results provide further evidence that senescence per se is much more resistant to radiation than might be inferred from studies in which the three typical radiation effects, mentioned in the introduction, contribute to damage. Some of the findings in this study have been extended to the ultrastructural level in the electron microscope study described in the immediately following report.[2]

References

[1]A. H. Haber, C. Scurry, and R. Gehring, *Biol. Div. Semiann. Progr. Rept. July 31, 1966,* ORNL-3999, pp. 161–62.

[2]P. L. Walne, S. A. Angel, and A. H. Haber, sect. 13.3, this report.

13.3 EFFECTS OF ACUTE GAMMA IRRADIATION ON SUBSEQUENT CHANGES IN CHLOROPLAST ULTRASTRUCTURE IN EXCISED, NONILLUMINATED WHEAT LEAF TISSUE

Patricia L. Walne Sarah A. Angel
A. H. Haber

Introduction. – Previous investigations[1] of the effects of acute gamma irradiation on excised wheat leaves have assumed that declining chlorophyll contents are an index of chloroplast deterioration. After receiving lethal doses, leaf tips kept in darkness showed a slower rate of chlorophyll decay and smaller losses of insoluble nitrogen than did either leaf tips kept in the light or unirradiated controls. This report describes a correlative electron microscope investigation to study radiation effects on chloroplast morphology and integrity at the ultrastructural level.

Results and Discussion. – Apical portions (2 cm) of wheat leaves were excised from one-week-old plants and were given 1 megarad of ^{60}Co gamma radiation in darkness. Another group of leaf tips served as non-gamma-irradiated controls. These 2-cm-long leaf tips were floated on 10^{-3} *M* potassium phosphate buffer at pH 6 in darkness for various times before fixation for electron microscopy. For controls some non-gamma-irradiated tissue was fixed immediately at the time of excision; some gamma-irradiated tissue was fixed immediately after cessation of gamma irradiation. In all cases, the tissue studied was chlorenchymatous mesophyll, approximately 1 cm from the leaf apex.

All tissues were fixed in 3% glutaraldehyde–3% acrolein buffered with 0.2 *M* sodium cacodylate. Aliquots were post-fixed either in 2% osmium tetroxide buffered with sodium cacodylate or in 2% unbuffered aqueous potassium permanganate. After dehydration in a graded series of ethanols, materials were embedded in a mixture of Araldite and Epon and were sectioned with a diamond knife on a Sorvall Ultramicrotome II. Sections were afterstained with uranyl acetate and lead citrate and were viewed with an RCA-3D electron microscope.

Chloroplasts of leaves fixed immediately after irradiation and of unirradiated controls fixed at the same time appeared no different from each other. However, examination of irradiated and control materials fixed after two days in darkness and again after five days in darkness revealed differences in chloroplast structure. After two days in darkness, chloroplasts of unirradiated controls evinced some vacuolation of lamellar elements, some apparent decrease in density of matrix material, an increase in size and number of dense, osmiophilic granules, and some general degradation of entire chloroplasts, due probably to breakdown of portions of chloroplast envelopes. After five days in darkness, chloroplasts of unirradiated controls were virtually nonexistent, with only occasional traces of large vacuolated lamellar elements and associated dense osmiophilic granules.

In marked contrast, chloroplasts of the irradiated leaf tissue after two days in darkness appeared nearly normal in structure and showed only slight vacuolation of lamellae and no observable difference in density of the matrix. After five days in darkness, irradiated chloroplasts in gamma-irradiated tissue evinced considerable degradation, but not as severe as that in non-gamma-irradiated tissue. Although some vacuolate lamellae were observed, the usual configurations of grana and stroma lamellae were discernible. Matrix material, though less dense, was identifiable, and large portions of the chloroplast envelope were intact. An increase in size and number of dense osmiophilic granules was observable, but in general, chloroplast structural integrity was not as markedly disrupted as in the unirradiated controls.

These results confirm the assumption that gamma-radiation-induced differences in chlorophyll decay in darkness are indeed correlated with corresponding differences in loss of chloroplast integrity at the ultrastructural level. Since the loss of chloroplast structure in darkness may be considered one aspect of senescence at the subcellular level, it is perhaps of developmental interest that this aspect of senescence can be suppressed by a lethal dose of irradiation. These findings are consistent with the findings suggesting that at the histological level also senescence is developmentally programmed, as described in the immediately following report.[2]

References

[1]A. H. Haber and W. D. Willis, sect. 13.2, this report.

[2]D. E. Foard, S. A. Angel, and C. P. Richardson, sect. 13.4, this report.

13.4 EVIDENCE FOR HISTOLOGIC PROGRAMMING OF SENESCENCE SEPARATE FROM CELL GROWTH AND DIVISION

D. E. Foard Sarah A. Angel
Carolyn P. Richardson

Introduction. – The usefulness of the gamma-plantlet (i.e., a wheat seedling growing without cell division after heavy gamma irradiation of the unsown grain) for investigations of senescence has been suggested in a previous report.[1] All organs of the gamma-plantlet are determinate; thus roots of gamma-plantlets do not have the capacity to grow indefinitely. Typically the apical region of an unirradiated, normal root has the potential to elongate indefinitely; moreover, basal regions of roots have the capacity to form branch roots from the pericycle. Associated with the elongation of the root, as well as with the formation of branch roots, are mitoses and cell divisions in the apical region and basally in the pericycle. Assuming that loss of capacity to make insoluble RNA is an indicator of senescence, in this report we describe a means to demonstrate in roots of gamma-plantlets the morphologic programming of senescence in the absence of the cell growth and division that normally occur in the apical growth in length of parent roots and the basal growth in the pericycle during initiation of branch roots. We have exposed lower lateral seminal roots of gamma-plantlets of various ages to tritiated uridine for 2 hr and observed the pattern of incorporation into insoluble RNA by means of autoradiographs of serial sections.

Results and Discussion. – Two and three days after sowing, lower lateral seminal roots of gamma-plantlets (500 kilorads) were increasing in size throughout their lengths. The labeling pattern in these roots was characteristic for RNA with 100% of the cells labeled, heavy nucleolar labeling, and cytoplasmic labeling. Four and five days after sowing, roots were still growing in length, although the most basal cells had stopped growing or were growing very slowly. Incorporation of the labeled uridine into insoluble RNA in these roots was found in all cells except the outermost root-cap cells and the most basal epidermis, cortical parenchyma, and vascular tissue. The only labeled cells in the most basal part of the roots were in the pericycle, the tissue in which branch roots are initiated. By 11 days after sowing, lower lateral seminal roots had stopped growing. Labeled cells occurred in the most proximal layer of the root cap and in all the parenchymatous cells of the apical 200 to 300 μ of the root proper. Basal to this apical zone, labeled cells occurred only in the pericycle, extending some 200 to 300 μ. In roots of gamma-plantlets 21 days after sowing, all parenchymatous cells of the apical 100 to 200 μ were labeled. Labeled cells in the pericycle may occur 100 to 400 μ basal to the labeled apical zone. By 27 days after sowing, roots of gamma-plantlets may have some labeled cells in the apical 100 μ of the root proper; some roots at this time are unlabeled.

These results show that histologic differences in senescence within the gamma-plantlet root parallel histologic differences in development within the normal, unirradiated root. Thus regions of gamma-plantlet roots corresponding to those regions of unirradiated roots normally "programmed" to perpetuate development are the ones that "outlive" those regions normally "programmed" to cease development. These results are evidence for histologic programming of senescence separate from cell growth and cell division.

References

[1] A. H. Haber, Pat C. Kerr, and D. E. Foard, *Biol. Div. Semiann. Progr. Rept. July 31, 1965*, ORNL-3853, pp. 177–78.

13.5 PHOTOSYNTHESIS

W. A. Arnold L. W. Jones
W. F. Bertsch R. M. Pearlstein
J. R. Azzi J. B. Davidson[1]

Photosynthesis is the process in which green plants reduce carbon dioxide to carbohydrate and oxidize water to oxygen. The energy comes from sunlight absorbed by chlorophyll. The time scales relating to fluorescence of chlorophyll in vivo, 10^{-9} sec, and to steady-state turnover time of the enzymatic systems, 10^{-2} sec, are seven orders of magnitude apart. This means that although the excitation energy was no longer freely able to excite chlorophyll within 10^{-9} sec after the initial absorption act, this energy did not appear as stabilized chemical products until 10^{-2} sec after absorption. It follows that in the intermediate time range there is a storage of energy in metastable forms. We have

developed several methods of studying this energy storage by measuring reemission of light from plants and plant fragments. The reemission is due to low-yield reexcitation of chlorophyll by the metastable stored energy. Work with mutant algae has shown that the reemission of light is due to energy storage by that part of the photosynthetic apparatus which has been defined as photoreaction II, the oxygen-evolving photoreaction.

Delayed Light Studies on Chloroplasts.[2] – The delayed light emission from angiosperm chloroplasts in the presence and absence of hydrogen acceptors has been measured from 1 to 20×10^{-3} sec after the centers of repeating flashes of exciting light. These experiments allow study of the interaction between energy storage and activation of the electron transport enzymes associated with photoreaction II at times earlier than the turnover time of the enzymes.

Extraction of chloroplasts from leaves resulted in millisecond dark decay kinetics which were very slow. These flat decay curves were quite unlike those observed in whole leaves or in whole algal cells, but resembled the decay kinetics previously observed from cells poisoned with DCMU[3] or from cells in which photoreaction I was nonfunctional due to mutation. Activation of noncyclic electron transport in chloroplasts by either $K_4Fe(CN)_6$, NADP/FD, DCPIP, or methyl viologen (pseudocyclic) resulted in increased intensity of emission at 1 msec and in more rapid dark decay of the emission, so that the shape of decay curves from activated chloroplasts approached that from whole leaves.

Heating of chloroplasts to 50°C for 5 min was shown to destroy the Hill-reaction capacity of photoreaction II without affecting the ability of photoreaction I to reduce NADP in the presence of FD with reduced DCPIP as the electron donor. These chloroplasts did not emit delayed light. This result confirms for angiosperms the earlier conclusion that algae emit delayed light due to energy storage by photoreaction II. This conclusion is strengthened by two other observations with chloroplasts. First, concentrations of DCMU which eliminated the Hill reaction had only a small effect on the millisecond emission in the absence of hydrogen acceptors, but eliminated the effect of adding the hydrogen acceptors. Second, only a small effect was noted on delayed light due to activation of cyclic electron transport by PMS or pyocyanine, both of which are cofactors of a cyclic phosphorylation which utilizes very little energy from photoreaction II.

We interpret these results in terms of modulation of energy storage by photoreaction II, due to activation of enzymatic electron transport in a two-photoreaction scheme. Activation of the enzymatic steps results in a transient increase in the energy which is stored at times somewhat faster than the turnover time of the enzymes and also results in more rapid utilization of this stored energy during the times that the enzymes are functioning. The increase in energy storage can be explained in terms of an electron-hole picture of the energy handling processes at the reaction center of photoreaction II. Acceptance of a reducing equivalent by an enzymatic process would result in a mobile hole being left in the reaction center; acceptance of an oxidizing equivalent by a different enzymatic process (spatially separated from the first) would leave a mobile electron in the reaction center. Thus activation of enzymatic acceptors would result in additional energy storage in the chlorophyll system, and this additional energy storage would persist until the charge which was left in the pigment system could be accepted by the proper enzymes. Delayed light emission would result from reexcitation of chlorophyll due to occasional charge recombination on occupation of a single chlorophyll molecule by both an electron and a hole.

Demonstration of the Biphotonic Nature of Delayed Light Emission from Whole Algal Cells. – The electron-hole hypothesis given above to explain the transient increase in energy storage due to activation of the enzymatic reactions of photoreaction II would also require that two different sites in the reaction center be energized before delayed light emission could take place. Thus both the delayed light emission and the evolution of oxygen should require two absorption acts in photoreaction II. We have found that the intensity of delayed light at 10^{-1} sec from cells previously kept in darkness varies as the square of the exciting light given as a flash. Joliot has given evidence that oxygen evolution, from cells similarly kept in darkness, requires two absorption acts in photoreaction II.

Energy Storage at Low Temperature. – Algal cells frozen to −196°C are able to store energy from a single flash of light, as shown by the reemission of light on heating the cells to 100°C. Such frozen cells also emit delayed light while in the frozen condition. The electron-hole picture predicts that the delayed light should not be emitted from frozen cells after the reaction centers are completely energized, since the enzymatic acceptors would be

nonfunctional and there would be no way for additional charge to be formed in the chlorophyll system. We have found that the delayed emission from frozen cells does die away after the first few flashes of exciting light. Furthermore, the light emission on heating shows that such cells were indeed charged.

Excitation of in Vivo Chlorophyll Fluorescence by Beta Particles. – It is clear that there is a need to study energy storage at times in the range of the lifetime of the in vivo singlet state of chlorophyll (10^{-9} sec), since we have no information at all about the nature of the storage mechanism which first accepts energy from the excited singlet. A first step in this direction would be to determine when the decay of fluorescence becomes nonexponential, and to determine what factors affect the kinetics of fluorescence decay. Since sufficiently short light flashes are not available, it is proposed to excite the chlorophyll by beta particles. Preliminary results on leaf plugs excited with a ^{147}Pm beta source indicate that light emission from plants may be excited by beta particles. The emission shows the spectral characteristics of chlorophyll fluorescence. It is hoped to develop this experiment in order to study the decay characteristics of in vivo chlorophyll fluorescence.

References

[1]Instrumentation and Controls Division.

[2]These experiments were conducted in the Biochemistry Department of the University of Cambridge in collaboration with Dr. Robert Hill and Dr. Janet West.

[3]Abbreviations:

DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenyl indolphenol
PMS	phenazine methosulfate
NADP/FD	triphosphopyridine nucleotide and ferredoxin.

Cell Physiology

Peter Mazur	Margaret R. Kasschau
S. P. Leibo	Janice J. Schmidt
J. F. Brandts[a]	Patricia F. Davis[b]
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14.1 FREEZING OF *ESCHERICHIA COLI*

Peter Mazur Janice J. Schmidt

The initial experiments reported previously[1] have been extended. Stationary phase cells of *E. coli* B/r ORNL were washed and suspended in 0.006 *M* phosphate buffer, distilled water, or 0.006 *M* NaCl, cooled to −196°C at rates ranging from 14 to 4200°C/min, and warmed slowly (1°C/min) or rapidly (1000 to 35,000°C/min). The chief results are summarized in Fig. 14.1.1.

Cells in Buffer or Water. –

1. As the cooling velocity was increased, survival rose to a maximum with cooling at 38°C/min and then dropped to a minimum at 560°C/min. A still higher cooling velocity (4200°C/min) produced no further shift in survival when warming was slow but yielded higher survival (72%) when warming was rapid. This higher survival could have been due to the fact that "rapid" warming in this case was 30 times higher (35,000°C/min) than in the case of the samples cooled at 14, 38, or 560°C/min, which warmed at 1000°C/min. The difference came about because the sample containers in the former case were thin capillary tubes.

2. Rapid warming yielded higher survival than did slow warming, and the difference became greater with higher cooling velocities.

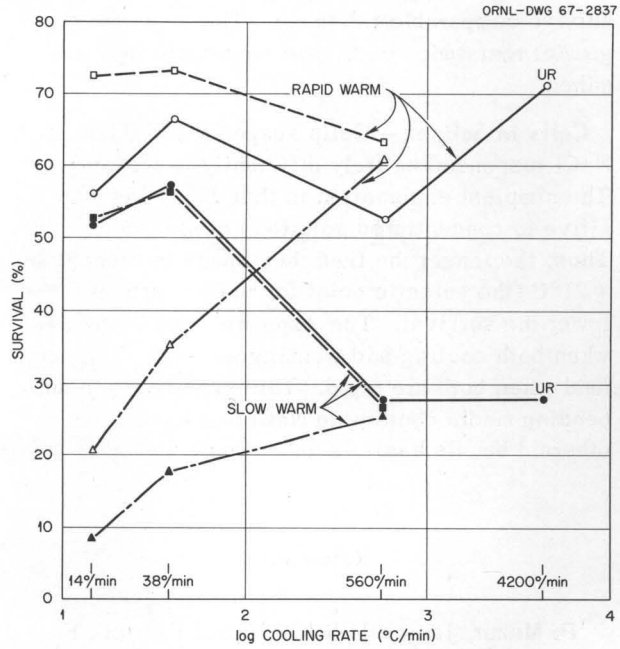

Fig. 14.1.1. Survival of Cells of *E. coli* B/r as a Function of the Cooling Velocity. Cells (1×10^8/ml) were suspended in water (——), 0.006 *M* phosphate buffer (– – –), or 0.006 *M* NaCl (–.–.–), cooled at various rates to −196°C, and warmed slowly (●,■,▲), or rapidly (○,□,Δ). Slow warming was 1°C/min; rapid warming was 1000°C/min except for the two points labeled UR, where it was 35,000°C/min.

3. These results are quite analogous to those obtained with yeast,[2] and we hypothesize that the same factors were involved; namely, slow cooling at 14°C/min produces slightly lower survivals than cooling at 38°C/min because it produces a longer exposure to the solutes that are concentrating during freezing. Increasing the cooling velocity shortens the exposure time, and hence survival rises. But when the cooling velocity rises above 38°C/min this beneficial effect of more rapid cooling is counteracted by the appearance of intracellular ice. The sequence of ultrarapid cooling and ultrarapid warming produces high survival, because it keeps the intracellular ice crystals from becoming injuriously large.

4. The results differ from yeast in two important respects: (*a*) The cooling velocity for optimum survival occurs between 38 and 560°C/min, whereas in yeast it occurs between 1 and 10°C/min. This was predicted because of the higher surface-to-volume ratio in *E. coli.*[3] (*b*) Some 30% of *E. coli* cells survive rapid cooling at 560 to 4200°C/min and slow warming, whereas only 10^{-6}% of yeast survive comparable treatment. The basis for the greater resistance of *E. coli* remains to be determined.

Cells in Saline. – Cells suspended in 0.006 *M* NaCl responded entirely differently to freezing. The simplest explanation is that *E. coli* is sensitive to concentrated solutions of sodium chloride. Thus, the longer the time they spend between 0 and −21°C (the eutectic point for NaCl solutions), the lower the survival. The exposure time is greatest when both cooling and warming are slow; it is minimal when both are rapid. This sensitivity to suspending media containing NaCl has been noted by others,[3] but its basis is unknown.

References

[1]P. Mazur, Janice J. Schmidt, and Patricia F. Davis, *Biol. Div. Semiann. Progr. Rept. July 31, 1966*, ORNL-3999, p. 129.

[2]P. Mazur and Janice J. Schmidt, *ibid.*, p. 129, Fig. 13.2.1.

[3]P. Mazur, in *Cryobiology*, ed. by H. T. Meryman, Academic, New York, 1966.

14.2 FREEZING OF BACTERIOPHAGE T4B

S. P. Leibo Peter Mazur

One of the following physical-chemical changes is undoubtedly responsible for the inactivation observed in frozen-thawed suspensions of bacteriophage T4B: (1) the temperature decreases, (2) ice crystals form, (3) liquid water is removed, (4) solutes are concentrated, (5) salt crystals form, or (6) interactions of the above changes occur. To distinguish between them, phage T4B suspended in water or various salt solutions has been cooled and warmed at controlled rates from subzero temperatures. The following conclusions may be drawn from recent results.

1. Decreased temperature by itself does not inactivate phage. As long as the suspension does not freeze, 100% of T4B in distilled water survives cooling and warming from −15°C, and 100% of T4B in 5.2 *m* NaCl survives cooling and warming from −40°C.

2. Ice crystal formation does not necessarily inactivate T4B. Approximately 95% of T4B suspended in 0.00052 *m* NaCl survives cooling at about 1°C/min and subsequent warming at the same rate from temperatures as low as −76°C. In this case, ice crystals are visibly present and the entire suspension must be frozen.

3. The removal of 99.999% of liquid water of the whole suspension by freezing does not inactivate phage. More than 85% of T4B suspended in 0.00052 to 5.2 *m* NaCl solutions survives rapid cooling and slow warming from −20°C, a temperature slightly above the eutectic point of aqueous NaCl solutions. Although the concentration of a solution is constant at a given temperature, at temperatures above the eutectic point the ratio of unfrozen to frozen is a function of the initial concentration of the solution. We observe that phage survival is essentially independent of that ratio, at least to the point at which only 0.001% of the volume of the entire suspension remains unfrozen.

4. Concentrated solutes do not inactivate phage T4B. The phage is completely stable in 1.15 *m* KNO_3, 3.0 *m* LiCl and $MgSO_4$, 3.3 *m* KCl, 4.6 *m* NH_4Cl, and 5.2 *m* NaCl. As long as neither salt crystals nor ice crystals are present, this statement is true in all cases at 25°C, and is also true between room temperature and −10°C (KNO_3), −20°C (KCl), and −30°C (NaCl).

5. The formation of salt crystals as water is removed as ice from a phage suspension may be responsible for the inactivation of T4B. Mazur[1,2] has observed by means of electrical resistance measurements that liquid is present in frozen salt solutions at temperatures almost 10°C below the eutectic point, a temperature at which complete solidification of salt and water should theoretically be observed. The discrepancy is due to supersaturation. We find that T4B suspended in eutectic concentrations of KNO_3, KCl, and NaCl is completely stable at subzero temperatures approximately 8°C below the eutectic point of the solution, even in the presence of ice, but that more than 90% of the phage is inactivated when exposed to temperatures 10°C or more below the eutectic point. This is so regardless of the cooling or warming rates. In other words, the phage are inactivated only when complete solidification of the dissolved salt has occurred. Moreover, decreasing the proportion of salt crystals in a frozen suspension by decreasing the initial concentration of the salt results in increased phage survival. This is shown by the results in Fig. 14.2.1. The inactivation of phage under these conditions may result from a competition for "bound water" between the salt and the phage or from an incompatibility between the structure of water ordered by the salt and that ordered by the phage. This suggestion is currently under investigation.[3]

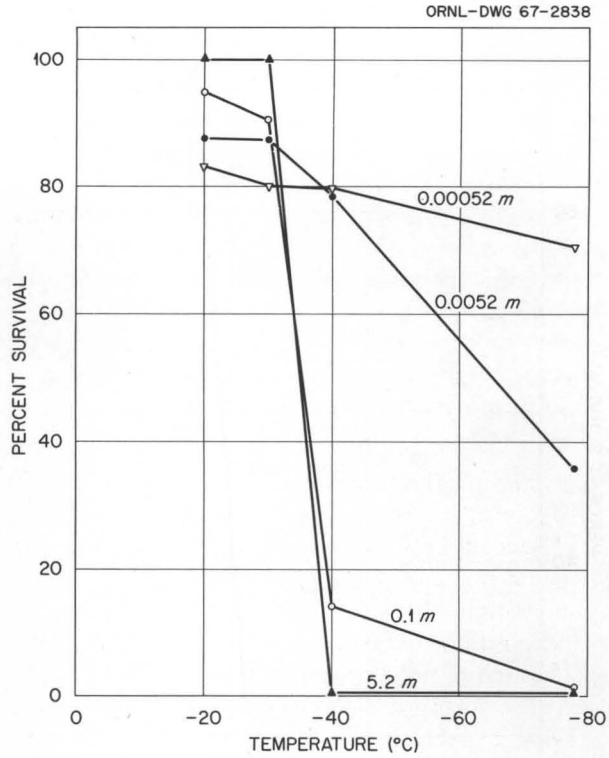

Fig. 14.2.1. Rapid Cooling–Slow Warming of T4B in NaCl Solutions.

References

[1]P. Mazur, *Biophys. J.* **1**, 247–64 (1961).
[2]P. Mazur, *Biophys. J.* **3**, 323–53 (1963).
[3]S. P. Leibo and P. Mazur, sect. 14.3, this report.

14.3 CORRELATION BETWEEN WATER ACTIVITY AND SURVIVAL OF BACTERIOPHAGE T4B

S. P. Leibo Peter Mazur

In the course of several experiments, we observed that bacteriophage T4B was inexplicably inactivated when suspended in concentrated solutions of some salts but not in others. By plotting phage survival as a function of the osmotic coefficients of the various solutions, a correlation became apparent. Extending this analysis, we plotted phage survival as a function of the water activity of the solutions, calculated from the expression

$$\ln a_w = \nu m M_w \phi / 1000 ,$$

where a_w is water activity, ν is the number of ions per molecule of electrolyte, m is molality, M_w is the molecular weight of water, and ϕ is the osmotic coefficient. The results in Fig. 14.3.1 show that phage T4B is completely inactivated when suspended in salt solutions whose water activity is less than 0.72, but is completely stable in solutions whose water activity is greater than about 0.80. Although T4B is also inactivated when suspended in 6.60 *m* NaBr and 4.26 *m* NaI, calculated water activities for these solutions are not included since corresponding osmotic coefficients are not available. However, a comparison of phage survival

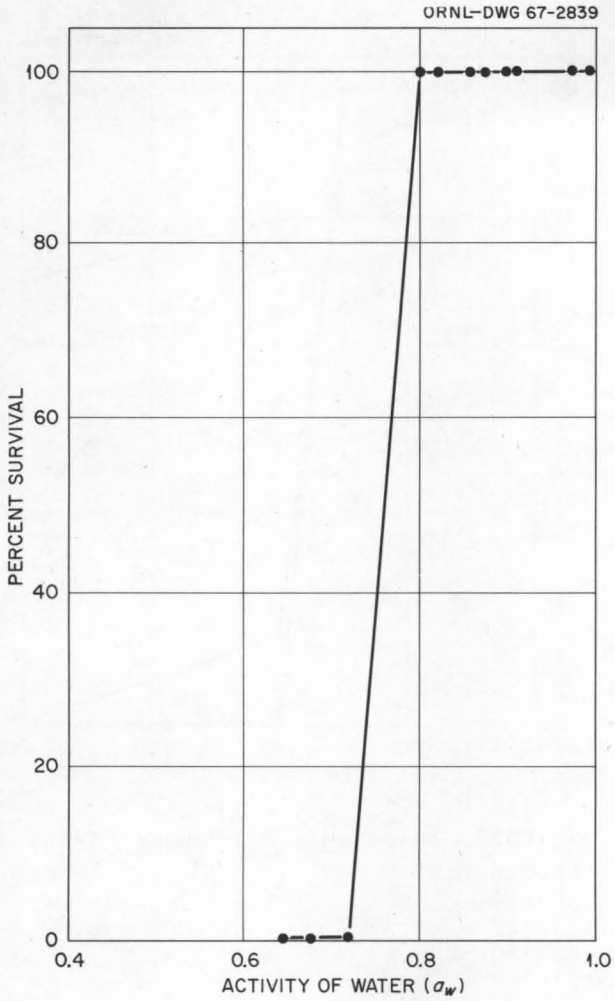

Fig. 14.3.1. Survival of Phage T4B as a Function of Water Activity of the Suspending Solution.

and the water activities of these two solutions, estimated by extrapolation, indicates that the correlation also holds for them.

Survival of the phage is independent of salt concentration, ionic strength, and the individual ions in solution, since T4B survives suspension in 3.0 *m* LiCl, 5.2 *m* NaCl, 6.0 *m* CsCl, and 3.0 *m* $MgSO_4$ but does not survive suspension in 6.0 *m* LiCl, 3.8 *m* $CaCl_2$, and 3.0 *m* $MgCl_2$ in addition to solutions of NaBr and NaI. Interpretation of these results must await further study.

14.4 SURVIVAL OF SUPERCOOLED YEAST

Peter Mazur Janice J. Schmidt
Margaret R. Kasschau

Fewer than 1 yeast cell in 100 million survives the sequence of cooling at rates above 100°C/min to below −60°C and slow warming at 1°C/min. The death of the majority has been shown to be due to the formation of intracellular ice.[1] However, even when cooling is low enough to preclude intracellular freezing (1°C/min), 50% of the cells are still killed below −30°C. The possible causes of the death of this 50% are: (1) Death is the result of a lowering in temperature. (2) Death is the result of ice formation and the concomitant concentration of solutes. (3) Death is a result of the interaction of ice formation and reduced temperature.

Ice formation itself has several consequences: It subjects the cells to the forces resulting from the phase transition (e.g., pressure and shear); it removes water from the cells by depositing it as ice; it, therefore, concentrates all solutes and decreases the spatial separation of macromolecules. Finally, if the temperature drops sufficiently low, ice formation eventually results in the precipitation of all cell solutes.

It is difficult to unravel the effects of these factors and the effect of temperature during the freezing of solutions (including protoplasm) 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. This restriction is removed in supercooled solutions. The present study is concerned, therefore, with determining the effects of temperature and solute concentration on the survival of supercooled suspensions of yeast.

Method. – Cells of *Saccharomyces cerevisiae* were suspended in distilled deionized water or in 2 *M* NaCl solutions. Before use, the suspending media were filtered through 0.45-μ Millipore filters. Small samples of the suspension (0.02 ml) were transferred by syringe to glass tubes and cooled at about 1°C/min to −18 to −20°C. With proper precautions, approximately half of the samples remained supercooled at these temperatures. After 10 min, the samples were warmed slowly at 1°C/min and their cell survival determined by plate count assay.

Results. – Preliminary results indicate that the survival of the cells in supercooled suspension remains close to 100% relative to room-temperature controls. However, intersample variability has been too high to permit a definitive statement about absence of injury. The survival in suspensions that froze at −18 to −20°C was 1% or less.

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Nucleic Acid Chemistry

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15.1 NUCLEOSIDE SEQUENCES IN TRANSFER RNA

Identification of Rare Nucleotides (Mayo Uziel, Chongkun Koh). – The rapid cation exchange analysis scheme for nucleosides has been improved with a new resin (BioRad A-6 in place of Amberlite CG 120, III, or Beckman AA-27) to permit detection, by separation, of pseudouridine, uridine, ribothymidine, inosine, 4-thiouridine, guanosine, *N*-dimethylguanosine, adenosine, cytidine, *N*-dimethyladenosine, 1-methylguanosine, and 7-methylguanosine, in that order. Several of these did not separate quantitatively in the previous scheme, which took about 1 hr. The present, more complete analysis requires 6 hr, largely because of the strong affinity for the cation exchanger of those nucleosides following cytidine. The bases of the major nucleosides, if present, also separate in the new scheme. However, some of the rare nucleosides (N^6-methyladenosine, N^2-methylguanosine, and 3-methylcytidine) are not separated from the major nucleosides.

The application of this higher resolution system to nucleoside mixtures derived from alkaline or enzymic hydrolysates of yeast or *E. coli* tRNA showed at least 17 different components.

Dihydrouridine, a component of tRNA that does not absorb uv light in the usual (240 to 300 mμ) range, must be detected by other means. A colorimetric test for ureido compounds, with an extinction coefficient of 9100, has been modified for use with tRNA. Yeast and *E. coli* tRNA's give values by this new test of 3.7 and 1.6 residues, respectively, per tRNA molecule.

Sequences in tRNA^{Phe} (*E. coli*) (Mayo Uziel, H. G. Gassen, A. Jeannine Bandy). – In addition to the T1 RNase (from Takadiastase) hydrolysates analyzed previously, we have begun the analysis of the RNase I (pancreatic ribonuclease) fragmentation of tRNA^{Phe} (*E. coli*). The number of bonds cleaved, measured in a pH-stat, is 29 ± 2, as expected on the basis of previous analyses. The largest fragment isolated appears to be a hexanucleotide of composition (G_3, A_2)U. The dinucleotides AC, GC, and GU have been identified.

15.2 OLIGONUCLEOTIDE INTERACTIONS

S. K. Niyogi

The interactions between nucleic acid materials are being studied in simplified systems in which one component is a synthetic oligo- or polynucleotide. The interaction of adenine oligonucleotides with polyuridylic acid (oligo A plus poly U) has been investigated[1] by measuring the temperature

at which the complex, absorbed on a hydroxylapatite column, releases the oligo A. This "melting" temperature is directly proportional to the chain length of the oligo A. Magnesium ion increases the "melting" (elution) temperature; therefore it increases the stability of the oligo A·poly U complex. From the proportionality between chain length and elution temperature, the melting temperatures of poly A·poly U in the presence and absence of Mg^{2+} can be estimated.

We have also sought to determine the minimum number of nucleotides required to form a specific, stable complex.[2] RNA synthesized in vitro on a T4 or T7 phage DNA template by RNA polymerase was degraded with T1 RNase (from Takadiastase) and fractionated according to chain length. The fractions were then annealed to denatured T4 or T7 DNA that had been immobilized on a nitrocellulose filter, and the complex was digested with RNase. A chain length of 10 or more was required to form a complex resistant to RNase, under the optimum conditions. The shortest oligonucleotides forming complexes did so with considerable species specificity; that is, T4-produced oligonucleotides complexed with T4 DNA only. A considerable degree of homology was observed between T3 and T7 and between T2 and T4 phage DNA's in that oligonucleotides produced by T7 would bind to T3 but not to T4 or T2, etc. Homology is known to be greater between two odds or two evens in contradistinction to an odd and an even phage DNA.

The stability of such homologous oligoribonucleotide-denatured DNA complexes has been investigated by the release of the oligoribo compound from the DNA-filter as a function of temperature. The "melting" temperature increased with the chain length of the oligo and also showed a dependence on its base composition.

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Nucleic Acid Enzymology

Elliot Volkin

Microbial Systems (16.1–16.5)

Elliot Volkin
W. E. Barnett
J. L. Epler
R. K. Fujimura
J. X. Khym
C. G. Mead
W. S. Riggsby[a]
Diane J. Goins
M. Helen Jones

Developmental Biochemistry (16.6)

F. J. Finamore
J. S. Clegg[a]
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16.1 PURIFICATION OF T2 MESSENGER RNA

Elliot Volkin W. S. Riggsby

Our early experiments with chloramphenicol revealed an interesting facet of the synthesis of messenger RNA, that is, that the prevention of protein synthesis blocks the turnover of messenger RNA. We are presently attempting to exploit this phenomenon for the purpose of accumulating and preparing relatively large quantities of T2-specific messenger RNA's. The addition of chloramphenicol some 6 min after infection with T2 allows the synthesis, without noticeable degradation, of some T2 messenger RNA but also allows the continued and vigorous synthesis of T2 DNA. By inserting an inhibitor of DNA synthesis (5-fluorodeoxyuridine) simultaneously with the addition of chloramphenicol, we can eliminate this source of massive competition for nucleic acid precursors and accumulate large amounts of what appears to be T2-specific messenger RNA (labeled "F" RNA). The "F" RNA has a gross nucleotide composition similar to the T2 DNA. It is of interest that the extent of isotope incorporated into RNA in these experiments is the same order of magnitude as that calculated from a T2-infected, uninhibited culture had there been no turnover of the messenger RNA. Hybridization experiments confirm that much, if not all, of the accumulated, labeled RNA anneals specifically to denatured T2 DNA.

"F" RNA was fractionated on a cellulose column using an ethanol-buffer gradient. RNA fractions obtained in this manner were tested for their ability to hybridize with T2 DNA immobilized on nitrocellulose disks. The amount of labeled RNA

which would bind to an excess of DNA varied from about 5% to about 40% among the cellulose column fractions. Those fractions which contained a high proportion of hybridizable label yielded saturation curves when high RNA/DNA ratios were used in the hybridization mixtures. The less efficient fractions did not yield saturation plateaus even with RNA/DNA ratios of 300 to 400. When plateaus are obtained, they represent hybridization of 40 to 50% of the immobilized DNA.

Unfractionated and partially fractionated "F" RNA preparations were further purified by adsorption to and elution from columns of DNA bound to nitrocellulose powder. More than 80% of this purified RNA is hybridizable with excess DNA on disks. The large capacity and relative simplicity of the column fractionation technique, together with the large amount of labeled RNA produced by the method described, may make possible the isolation of large amounts of genetically specific RNA.

16.2 REPAIR OF PARENTAL T5 BACTERIOPHAGE DNA AFTER INFECTION OF *ESCHERICHIA COLI*

R. K. Fujimura Elliot Volkin

Meselson[1] has presented evidence that, in the case of λ phage, recombination occurs by breakage and reunion without DNA replication, but he has some indication that new synthesis of polynucleotide may be necessary to join the breaks. The length of newly synthesized polynucleotide is probably short and should have little, if any, effect on the density of DNA. T5 has a higher recombination frequency than λ phage, and the DNA is not glucosylated as are the T-even phages; thus T5 may be a better subject for biochemical investigation than either λ phage or the T-even phages. We have looked for the repair of parental DNA in T5-infected cells and its possible involvement in recombination of DNA molecules. For this purpose we used a T5 mutant (T5ts 53) which produces a temperature-sensitive DNA polymerase. A culture of *E. coli* F was grown in a medium containing ^{32}P and BUdR and shifted to a higher temperature just before infection with T5. All ^{32}P-labeled *E. coli* and newly synthesized T5 DNA, if any, will be labeled with BUdR, and thus denser than infecting parental T5 DNA. Under these conditions a low but significant amount of ^{32}P incorporation into parental T5 DNA has occurred that could be identified after recentrifugation of parental DNA band in an alkaline CsCl density gradient. This should be due to repair of DNA probably without using the T5 DNA polymerase. According to Abelson and Thomas,[2] however, there are natural nicks in T5 DNA which are repaired upon infection of bacteria. Thus the ^{32}P incorporation observed may be the repair of these nicks, but the nicks, in turn, may be the "hot spots" of recombination observed by Fattig and Lanni.[3]

Another possible explanation is that the ^{32}P-labeled material is the *E. coli* DNA. This is still being investigated even though *E. coli* DNA is extensively degraded in the infected system.

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16.3 AN ENDONUCLEASE FROM T2-INFECTED *ESCHERICHIA COLI*

C. G. Mead

In the course of purification of the oligodeoxyribonucleotide transferase system from extracts of T2-infected *E. coli* cells, a DNA endonuclease activity has consistently been detected after each step in the purification procedure. After step 4, the elution from hydroxylapatite, no detectable exonuclease activity is present whereas the endonuclease activity remains. Some of the properties of this activity have been examined by following the reaction by means of sucrose density gradient centrifugation using labeled native T2 DNA as a substrate. The endonuclease has no heavy-metal requirement and is thus distinct from endonuclease I of *E. coli*.[1] ATP is not required as it is in the oligodeoxyribonucleotide transferase reaction. The most interesting property of this enzyme is with respect to its product. With time of incubation the product appears as a distinct slower-sedimenting band in a sucrose density gradient and is quite distinct from the sedimentation profile of the substrate DNA. Furthermore, there appears to be a lack of intermediate-sized pieces at early times of incubation or smaller-sized pieces after extended

incubation with the enzyme. Further work is in progress on characterizing this endonuclease activity and to determine whether it is involved in the oligodeoxyribonucleotide transferase reaction.

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16.4 MITOCHONDRIAL SPECIFIC TRANSFER RNA'S AND AMINOACYL-RNA SYNTHETASES

W. E. Barnett D. H. Brown[1]
J. L. Epler Diane J. Goins

Neurospora mitochondria have been isolated by sucrose gradient zonal centrifugation and analyzed for the presence of transfer RNA's (tRNA's) and aminoacyl-RNA synthetases. The following observations have been made: (1) Mitochondria contain tRNA's and synthetases for all 19 amino acids tested; (2) the mitochondrial aspartyl-, phenylalanyl-, and leucyl-RNA synthetases were found to be distinct from those found in the cytoplasm on the basis of chromatographic properties and specificities in acylation, that is, mitochondrial synthetases acylate mitochondrial tRNA's but not those from the cytoplasm; and (3) the mitochondrial aspartic acid, phenylalanine, and leucine tRNA's were also found to be distinct from those found in the cytoplasm.

We are currently examining in detail the mitochondrial synthetases and tRNA's for other amino acids in order to ascertain whether, as the results obtained so far indicate, all of the tRNA's and synthetases present in mitochondria are unique to this organelle.

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[1]Enzymology section.

16.5 THE USE OF THE CETYLTRIMETHYLAMMONIUM CATION IN NUCLEOTIDE SEQUENCE ANALYSES

J. X. Khym Mayo Uziel[1]

A terminal nucleoside of a polyribonucleotide releases its purine or pyrimidine base through reaction of the nucleoside with periodate in the presence of an amine. After such a reaction the polyribonucleotide now contains a 3′-terminal phosphate group. Treatment of this 3′-phosphomonoester end group with alkaline phosphatase yields a compound having a new terminal nucleoside. By repeating the reactions in a cycle, the base sequence of a polyribonucleotide can be determined. Neu and Heppel have determined optimum conditions for this stepwise cleavage reaction involving the release of a terminal base.[2]

Our aim is to use this method in sequence studies on transfer ribonucleic acid (tRNA). In higher-molecular-weight polyribonucleotides such as tRNA, the separation of the parent product from its released base involves several complicated manipulations. We have been investigating the use of the cetyltrimethylammonium (CTA) cation to simplify this step in the procedure.

The CTA salt of tRNA is soluble in 1 *M* chloride solutions and insoluble in 0.2 *M* chloride solutions. This is the property that is being used to separate quantitatively the parent product from its released terminal base in the stepwise degradation procedure. The following is a typical example of this method now being developed. After incubation at 46° in 1 *M* lysine monochloride–0.05 *M* $NaIO_4$ at pH 8.0, the CTA salt of tRNA is precipitated by the addition of water. After a few minutes of centrifugation, the terminal base is recovered in the supernatant fluid. The parent product, now containing a 3′-terminal phosphate group, is dissolved in 1 *M* lysine monochloride at pH 8.5 and is then treated with alkaline phosphatase. After a short incubation period, the tRNA-CTA salt is

again recovered by dilution with water. The cycle is repeated by dissolving the precipitate in the lysine-IO_4^- solution, which liberates a new terminal base. Standard analytical methods are being modified to give quantitative data on the extent of the release of terminal base in each cycle.

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16.6 BIOSYNTHESIS OF ADENINE DERIVATIVES BY NAUPLII OF *ARTEMIA SALINA*

F. J. Finamore Rose P. Feldman

Introduction. – Recently[1] we have found that nauplii of the brine shrimp, *Artemia salina*, are incapable of synthesizing purines de novo, but, as differentiation proceeds, total adenine increases at the expense of total guanine. Furthermore, P^1,P^4-diguanosine tetraphosphate appears to serve as a direct precursor of ATP, with its guanine moiety being converted to adenine in the transition.

To gain further insight into the mechanics of the conversion of guanine to adenine, we have exposed nauplii to a variety of purine derivatives labeled with ^{14}C and have measured their conversion to adenine.

Methods. – Nauplii obtained under sterile conditions were placed in small covered dishes which contained 5 ml of sterile artificial seawater and either 4-amino-5-imidazole carboxamide-^{14}C (specific activity 23 mc/millimole), ^{14}C-xanthosine 5′-phosphate (specific activity 30.6 mc/millimole), ^{14}C-inosine 5′-phosphate (specific activity 30 mc/millimole), or ^{14}C-guanosine 5′-phosphate (specific activity 42 mc/millimole) was added to a final concentration of 2 μc per milliliter of incubation medium. After incubation at 30° for 4 hr the nauplii were collected, washed, and the acid-soluble fraction prepared. Free purines were obtained from this fraction by acid hydrolysis and were separated by column chromatography using Dowex 50-H^+.

Results and Discussion. – Our preliminary investigations indicate that the above precursors are converted to adenine in the following relative order of effectiveness:

IMP >> GMP >> XMP

= 4-amino-5-imidazole carboxamide .

The results suggest that, in the conversion of guanine to adenine, hypoxanthine may be an intermediate and that a reductase similar to that discovered by Mager and Magasanik[2] may be active in converting guanine to hypoxanthine. Furthermore, the fact that hypoxanthine is readily converted to adenine indicates that adenylosuccinase is functional in the nauplii.

We are currently engaged in a search for both adenylosuccinase and GMP reductase in the nauplii and are attempting to determine the entire sequence and metabolic regulation in the conversion of guanine to adenine.

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Chemical Protection and Enzyme Catalysis

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17.1 CHEMICAL PROTECTION

D. G. Doherty Margaret A. Turner

We have been interested in the mechanism of action of the naturally occurring sulfhydryl peptide L-glutathione. In order to determine whether any enzyme mechanisms or specific surface binding steps were involved in either its radiation protective activity or its ability to detoxify other sulfhydryl compounds, we have synthesized the other optical isomers of glutathione, for example, γ-D-glutamyl-L-cysteinylglycine, γ-D-glutamyl-D-cysteinylglycine, γ-L-glutamyl-D-cysteinylglycine, and the alpha peptide L-isoglutathione. All of these peptides were equivalent in radiation protective activity to L-glutathione when injected intraperitoneally into $BC3F_1$ mice 15 min prior to a lethal (900 r) dose of x radiation. Their detoxification activity with other sulfhydryl compounds was also the same as glutathione. Thus it seems that glutathione is acting as a nonspecific sulfhydryl compound, as none of the isomers would have the same binding properties on either cell or enzyme surfaces.

17.2 GLYOXALASE I

D. G. Doherty Margaret A. Turner

Although widely present in biological systems, the role of glutathione in physiological processes is still relatively obscure. It has been definitely implicated as a coenzyme in four and possibly in two additional enzyme systems. In these its chemical properties of reaction with a carbonyl or olefinic group are involved, and its most striking property of oxidizability is of no importance. In order to further examine glutathione's role as a coenzyme we have chosen to investigate the glyoxalase I system. The glutathione optical isomers that were prepared for radiation protection studies are particularly suitable for this purpose. All of the glutathione isomers formed hemimercaptals with the substrate for glyoxalase I – for example, methylglyoxal. However, in the presence of the enzyme only L-gluthione and γ-D-glutamyl-L-cysteinylglycine gave rise to the formation of the thiol ester lactoyl glutathione. The γ-D-D and L-D isomers were totally inactive, as was, contrary to a previous report, the alpha isomer L-isoglutathione. The rate with the D-L isomer was approximately 60% of that with glutathione. Thus it seems that the L-cysteine configuration is essential for the glutathione peptides to function as a coenzyme. Kinetic and inhibition studies are currently in progress to determine the nature of this binding to the enzyme and gain some insight as to the active center of glyoxalase I.

Enzymology

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Protein Synthesis (18.1–18.6)

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Enzymatic Mechanisms in Protein Synthesis (18.7–18.10)

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Mammalian Cell Regulation (18.11, 18.12)

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Participation in Cooperative Programs:
AEC-NIH Chemical Carcinogenesis (22.2–22.7)

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18.1 COMPARISON OF MITOCHONDRIAL AND CYTOPLASMIC tRNA FROM *NEUROSPORA CRASSA*

D. H. Brown G. David Novelli
W. E. Barnett[1]

Introduction. – This is a continuing investigation to determine whether mitochondria have a system for synthesizing protein that is completely independent of the cytoplasmic system. In a previous report[2] we described a method for the large-scale preparation of mitochondria from *Neurospora crassa* and of the isolation of RNA from the mitochondria. We use aminoacyl RNA synthetases from mitochondria and from the cytoplasm to aminoacylate their respective tRNA's.[3] The two species of tRNA are charged with the same amino acid but with a different radioactive label. The two samples of charged tRNA are mixed and cochromatographed on the reverse-phase column developed by Kelmers *et al.*[4]

Results. – To date we have examined leucine tRNA with this procedure. The results indicate that of the five leucyl-tRNA's obtained from a whole-cell preparation, two species are cytoplasmic in origin and three species are mitochondrial. The salt gradient used (0.4 to 0.7 *M* NaCl) resulted in the complete separation of the cytoplasmic leucyl-tRNA from the mitochondrial leucyl-tRNA.

We intend to systematically explore the possible differences in the other specific tRNA's with this method.

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18.2 BIOCHEMICAL PROCESSING. LARGE-SCALE FERMENTATION

E. F. Phares Mary V. Long
G. David Novelli

The major activity with the large fermentation system has continued to be *E. coli* production for the joint tRNA project. One approach to circumventing phage infection in the extremely sensitive *E. coli* B was to employ batch fermentation on a two-shift-per-day basis. This method allowed reduced manpower and took advantage of the high cell concentrations possible in the system. In one particular run, a yield of 63 g of wet cells per liter was obtained in a culture which apparently still was in the log phase of growth.

The production of Neurospora on the 100-gal scale was accomplished with excellent results. Compared with growth in 10-liter flasks, mycelia were obtained in two-thirds the time with considerably higher content of mitochondria, a result of the excellent growth conditions in the large tank. A B-4 zonal ultracentrifuge (on loan from the Chemical Technology Division) has been set up in the pilot plant to aid in isolation of these mitochondria.

A scale-up of tissue culture for the L-cell project has been carried out successfully in a modified New Brunswick 14-liter fermentor. With the latter as a model, a special lightweight tissue culture apparatus has been fabricated.

The production operations are summarized in Table 18.2.1.

18.3 STUDIES ON ANTIBODY BIOSYNTHESIS

C. J. Wust

In a previous report[1] it was postulated that the synthesis of rat 7S gamma globulin (IgG) was under the control of at least two genetic loci, as has been demonstrated by others in humans and rabbits. The subunits of a molecule of IgG consist of two heavy (H) and two light (L) polypeptide chains of approximately 20,000 molecular weight for L and 50,000 for H. The proposal was suggested that the translation of genetic information for the L and H chains was accomplished on different classes of ribosomal aggregates. Since that report, Scharff reported findings that verify this hypothesis.[2] We have continued the studies with rat IgG synthesis.

Fraction C-2 (gamma$_2$ globulin) was prepared from rats and the subunits isolated as previously described.[1] Antisera to the H and L chains were made in rabbits and their C-2 fractions isolated. This serum component was labeled with ^{125}I and used as a reagent to be added to ribosomal preparations obtained from rat spleens.[3] The addition of ^{125}I-labeled anti-rat globulin L chain to ribosomal suspensions before final isolation and washing was made. Following centrifugation of these ribosomes through sucrose density gradients, samples were examined for ^{125}I using a Packard well-type gamma counter. Radioactivity was found in the region of aggregates of five to nine ribosomes with the peak at six to seven. When the samples were examined for specific activity (counts min^{-1} OD$^{-1}_{260\ m\mu}$), discrete peaks were found with a tendency to be shifted from the optical density peaks to a slightly faster sedimentation.

The ability of ^{125}I-labeled anti-rat L chain to combine with these ribosomes was determined at 4-hr intervals after the injection of antigen (sheep erythrocytes) into the rats. At 8 to 12 hr after injection, the level of combination is about one-third of that found in control animals and at 24 hr

Table 18.2.1. Summary of Cell Production

Organism	Runs	Volume (liters)	Yield (kg of wet cells)	Group	Cell Components to Be Recovered
E. coli B	8	2400	140	Chem. Tech. (Enzymology)	tRNA
E. coli B	2	400	24	Enzymology	Miscellaneous
E. coli B, T2-infected	2	600		Enzymology	Phage-induced tRNA
Photobacterium phosphoreum	3	980	4	Univ. of Georgia	Luciferase
Photobacterium fischeri	2	700	6	Univ. of Georgia	Luciferase
E. coli B/N	1	20	0.26	Enzymology	RNA polymerase
Desulfovibrio gigas	2	800	0.76	Univ. of Georgia	Electron transport system
Neurospora crassa	4	1250	36	Nucleic Acid Enzymology and Enzymology	Mitochondrial tRNA
Clostridium pasteurianum	1	400	1.5	Biophysics	Ferredoxin
S. cerevisiae	1	200	1.8	Yeast Genetics	

after injection. The latter two values were not significantly different. This phenomenon is believed to indicate a transition period from "normal" globulin translation to "immune" globulin translation and the beginning of the production phase of the immune response.

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18.4 STUDIES ON RNA DURING THE IMMUNE RESPONSE

C. J. Wust D. G. Ridner

Introduction. – The analyses of various RNA's isolated from mammalian tissue and microorganisms suggest that regulation of protein biosynthesis may be the result of the quantity and activity of certain RNA species. The role of mRNA is well established, and evidence is accumulating to indicate that the specificity of tRNA may be a factor in control mechanisms. During the immune response nucleic acid synthesis increases concurrently with the proliferation of particular cell types and the production of antibody. Analyses of ribosomal and soluble RNA synthesized during the immune response has been initiated.

Results and Discussions. – Previous studies with ribosomes[1] showed that ribosomes could be isolated from rat spleen either by altering the ionic strength of the medium or by the use of sodium deoxycholate. The 10,000 × *g* pellet of the homogenate contains unbroken cells, intact nuclei, and tissue debris. Attempts to isolate ribosomes from this pellet by direct treatment with deoxycholate resulted in the release of fibrous DNA from which little if any material could be further fractionated. We have found that 1% Triton X-100 treatment of this pellet releases a quantity of ribosomes that can be isolated and analyzed.

Ribosomes obtained from the 10,000 × *g* pellet when analyzed by centrifugation in sucrose density gradients show a large amount of polysomal aggregates. The optical density profiles of these ribosomes are distinctively different from those obtained during routine isolation.

RNA has been isolated from ribosomes using methods previously described[1] (cytoplasmic ribosomes) and from those released by treatment of the 10,000 × *g* pellet with Triton X-100. The typical profile after centrifugation through a 9 to 36% glycerine gradient reveals two characteristic patterns for the two preparations. The profile of the cytoplasmic ribosomes shows the usual small amount of 4S and large amounts of 16S and 28S in addition to a distinct but small peak having a sedimentation greater than 30S. Profiles of RNA from ribosomes of the 10,000 × *g* pellet show the 4S, 16S, 28S, and a pronounced peak at a sedimentation greater than 30S. The latter peak is approximately two-thirds that of the 16S. In addition, there is a suggestion of a fifth peak of sedimentation greater than 40S. These preparations have little amino acid acceptor activity in a cell-free system. They are presently being studied in their role in stimulating amino acid incorporation onto ribosomes. In preliminary experiments, no differences could be found in the profiles and yields between "normal" and "immune" ribosomes.

Transfer RNA has been isolated from spleen homogenates and is under current study. It is of interest that little tRNA can be isolated from a 105,000 × *g* supernatant fluid from spleen, in contrast to the amount isolated from liver.

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18.5 ACTIVITY STUDIES ON *E. COLI* POLYSOMES

Peter Pfuderer Donna G. Shaffer

Polysomes produced by the addition of small amounts of actidione to a relaxed mutant of *E. coli* K-12 are currently being characterized by their ability to incorporate amino acids under various conditions. These polyribosomes have previously been shown to consist of long lacelike networks containing of the order of 50 to 100 ribosomes for the larger species. The smaller polyribosomes, containing from 1 to 10 ribosomes, are very similar in appearance to mammalian polyribosomes, that is, appear "clustered" under appropriate conditions in the electron microscope. The activity of sucrose density gradient fractions of these polyribosomes to synthesize protein was measured. They can apparently be divided into classes by their ability to use their own endogenous messenger RNA or to respond to a synthetic message. Whether this is due to actual differences in polyribosome structure, to a repressor functioning at the polysome level, or simply some messages being read faster than others is not known.

18.6 STUDIES OF THE BINDING OF RNA POLYMERASE TO POLYNUCLEOTIDES

Audrey L. Stevens

The binding of highly purified RNA polymerase from *E. coli* to different polynucleotides has been studied.

Earlier experiments had shown that binding of ^{3}H-labeled enzyme to native lambda DNA was not affected by temperature or presence of ribonucleoside triphosphates but was very sensitive to ionic strength. The maximum amount of enzyme bound was 2 μg per microgram of lambda DNA, or about 80 molecules per molecule of lambda DNA. Further studies have shown that enzyme bound in the absence of ribonucleoside triphosphates is exchangeable with free enzyme or free template, while enzyme bound in the presence of ribonucleoside triphosphates (a condition allowing RNA synthesis) is irreversibly bound. That each bound enzyme molecule may be capable of initiating RNA synthesis is suggested by the finding that the maximum number of RNA molecules that can grow on a molecule of lambda DNA is about 100.

Further studies of the binding of RNA polymerase to tRNA have been carried out. The binding reaction shows the same general features as the DNA-enzyme reaction – that is, it proceeds as well at 0 as at 37° and is unaffected by metal. Two complexes of tRNA and enzyme can be isolated by density gradient centrifugation. One is a 16S complex, the other a 20 to 22S complex. The 20 to 22S complex shows the sensitivity to ionic strength of the DNA-enzyme complex, while the 16S complex is considerably more stable. Certain studies of the two complexes suggest that they represent two different forms of the enzyme, both binding one or two molecules of tRNA. Analyses to see if these two forms are the previously de-

scribed monomeric and dimeric forms are currently being carried out.

18.7 THE BINDING OF $tRNA^{Phe}$ TO PHENYLALANYL-RNA SYNTHETASE

M. P. Stulberg K. R. Isham

Introduction. – The number of binding sites in tRNA for aminoacyl-RNA synthetases is unknown, although it can be assumed that the recognition site and the –CAA terminus are responsible for part or all of the interaction. We are using the technique of inhibition of phenylalanyl-RNA formation by various altered $tRNA's^{Phe}$ (ref. 1) in an attempt to determine the total number of binding sites in $tRNA^{Phe}$ for phenylalanyl-RNA synthetase.

Results and Discussion. – Periodated $tRNA^{Phe}$ and $tRNA^{Phe}$ that has been hydrolyzed to various limit residues with venom phosphodiesterase have been examined kinetically for their inhibitory capacities:

Treatment	K_i
Periodation	6.1×10^{-7}
4.6% hydrolysis	8.2×10^{-7}
46% hydrolysis	3.1×10^{-6}

By a comparison of the inhibitor constants it can be concluded that (1) the intact –CCA terminus (difference between the periodated and 4.6% hydrolyzed treatments), (2) the sequence between 4.6% hydrolyzed (elimination of –CCA terminus) and 46% hydrolyzed tRNA, (3) the remaining inhibition (recognition site) in the 46% hydrolyzed residue all contribute to the binding of $tRNA^{Phe}$ to the synthetase. The inhibition represented by the undefined sequence between the 4.6 and 46% hydrolyzed residues could also be interpreted as being due to a more favorable conformation for binding to the enzyme rather than an additional binding site. Once the recognition site has been destroyed by further hydrolysis,[1] no inhibition is observed by the remainder of the molecule.

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18.8 EFFECT OF METHYLATION ON PHENYLALANINE TRANSFER RNA FROM *ESCHERICHIA COLI*

L. R. Shugart M. P. Stulberg

Introduction. – In a continuing effort to obtain preparations of tRNA[1] in a high state of purity, we have reinvestigated the technique of reversed-phase column chromatography as a tool for the separation of $tRNA^{Phe}$ from mixtures of tRNA's synthesized by an RC^{rel} mutant of *E. coli* before and after methionine starvation.

Results and Discussion. – Initial separation of normal and undermethylated $tRNA^{Phe}$ has been accomplished by reversed-phase column chromatography with a type I organic phase[2] (dimethyldilaurylammonium chloride in isoamylacetate). Rechromatography of the $tRNA^{Phe}$ fractions on similar columns with a type II organic phase[3] (tricaprylylmethylammonium chloride in tetrachlorotetrafluoropropane) has resulted in further resolution and purification of normal and undermethylated $tRNA^{Phe}$. The various species of $tRNA^{Phe}$ obtained are being characterized by their (1) degree of methylation, (2) amino acid accepting capacity, and (3) end group base analysis. Initial studies based on total amino acid acceptance and the rate of reaction indicate that the undermethylated species are 50 to 75% as reactive as normal $tRNA^{Phe}$.

After in vitro methylation and isolation of methyl-deficient $tRNA^{Phe}$, a decay in phenylalanine acceptance activity has been noted. This has been tentatively attributed to insufficient removal of contaminating nucleases present in the methylating enzyme preparation. Attempts are under way to isolate the in vitro methylated $tRNA^{Phe}$ by fast separation from the nucleases with DEAE chromatography.

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18.9 LARGE-SCALE ISOLATION OF PHENYLALANYL-RNA SYNTHETASE FROM *E. COLI* B

R. L. Pearson[1] M. P. Stulberg

Introduction. – The adaptation of the method of isolation of phenylalanyl-RNA synthetase to large-scale procedures has been largely completed after the introduction of several modifications in the procedure.[2] A relatively large-scale isolation has been accomplished, and the experience gained should allow us to initiate routine pilot-scale preparations of this enzyme and other aminoacyl-RNA synthetases.

Results and Discussion. – We have discovered that impurities in the final enzyme product can be largely attributed to enzyme that has been denatured under the unavoidable conditions of large-scale procedures. The denatured protein can easily be separated from the active enzyme by DEAE chromatography. Therefore this fractionation has been introduced as a final step in the enzyme purification. The use of DEAE chromatography for concentrating the more pure but dilute fractions of the enzyme has been investigated and has proved to be a valuable adjunct to pervaporation-dialysis techniques.

We now have available sufficient quantities of materials to prepare large fractionation columns and anticipate a complete production run without limitations on any one fraction size.

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[1]Chemical Technology Division.

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18.10 OPTICAL STUDIES OF THE STRUCTURE AND FUNCTION OF tRNA

M. P. Stulberg J. W. Longworth[1]

Introduction. – The availability of pure preparations of $tRNA^{Phe}$ and phenylalanyl-RNA synthetase has made feasible the use of optical rotary dispersion (ORD) to examine the conformation of the tRNA molecule and the use of specific fluorescence techniques in studying the interaction of these macromolecules.

Results and Discussion. – The combination of synthetase and tRNA produces a fluorescence quenching at 335 mμ that is attributable to changes at the tryptophan residues in the protein. This phenomenon seems to be specific for $tRNA^{Phe}$ and requires the presence of ATP, Mg^{2+}, and phenylalanine. The relation of the reactivity and tertiary structure of $tRNA^{Phe}$ is being examined by ORD. Preliminary results indicate a dependence on an unaltered –CCA terminus for the maintenance of the normal conformation and full reactivity of the molecule.

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18.11 ISOENZYMES: THEIR REGULATION AND GENETIC CONTROL

K. B. Jacobson J. B. Murphy

Introduction. – The alcohol dehydrogenase of *Drosophila melanogaster* exists in five electrophoretic forms. Since they appear to be under control of a single genetic locus it is suggested that a common protein moeity exists for each isoenzyme and that their electrophoretic differences are the result of ligand binding or conformational reorganization.

Results. – After treating a homogenate with *n*-butanol the alcohol dehydrogenases may all be adsorbed onto DEAE-cellulose. The isoenzyme form that is eluted seems to be a function of the length of time the enzyme was in contact with the DEAE-cellulose. When elution by a salt gradient took 12 hr, 95% or more of the activity emerged as ADH_5. If the elution was carried out in 4 hr the ADH_5 constituted 70% of the eluted activity and ADH_3, 30%, with an overall recovery of 90% of the enzyme applied to the column. This demonstrates an interconversion of ADH_3 to ADH_5 and thus is direct evidence for the common protein moeity existing for each of these two isoenzymes.

The two major isoenzymes, ADH_3 and ADH_5, may be distinguished further on the basis of thermal inactivations; ADH_3 is stable at 37°, whereas ADH_5 can be completely inactivated in an hour at this temperature. To characterize the fractions obtained by DEAE-cellulose chromatography the stability to heating was examined. A fraction

consisting of ADH_3 was rechromatographed and allowed to convert to ADH_5. Testing the ADH_5 derived from ADH_3 for thermal inactivation showed that it was inactivated. Therefore, by the criteria of electrophoresis, chromatography, and thermal inactivation, not only can ADH_5 be distinguished from ADH_3 but the ADH_5 derived from ADH_3 is similar to the ADH_5 normally present. Therefore the direct interconversion has been substantiated.

Discussion. – With regard to the tertiary structure of ADH_3 and ADH_5, the transition from a thermal-stable to a thermal-unstable form along with the change in charge configuration will be given some attention. We cannot yet state whether a change in ligand binding or configurational rearrangement is the mechanism for alteration, but either will be of interest. Of further interest will be the exploration of the effect of butanol treatment, since the enzyme does not adsorb to DEAE-cellulose unless so treated; yet no alteration of the electrophoretic behavior results from this treatment.

18.12 FURTHER STUDIES ON THE AMINOACYLATION OF *E. COLI* tRNA FRACTIONS BY *NEUROSPORA CRASSA* PHENYLALANINE tRNA SYNTHETASES

Virginia L. Holten K. B. Jacobson

Introduction. – Previous observations demonstrated that a phenylalanine tRNA synthetase from *Neurospora crassa* could phenylalaninate the tRNA's of *E. coli* that normally accepted valine and alanine. In the present study attention is focused on the maximal amount of phenylalanine that can be esterified to these species of tRNA.

Methods. – The phenylalanine-tRNA synthetase of *Neurospora*, the valine-tRNA synthetase of *E. coli*, and the alanine-tRNA synthetase of *E. coli* were characterized for optimal activity with $tRNA_{coli}$ as a function of pH, magnesium concentration, ATP concentration, enzyme concentration, phenylalanine concentration, and NaCl concentration.

The fractionation of $tRNA_{coli}$ on "Freon columns" was performed by J. Weiss (Chemical Technology Division), who kindly made fractions available to us.

Results. – In an unfractionated sample of $tRNA_{coli}$ the amounts of amino acid accepted from *E. coli* enzyme were: valine, 19.63; alanine 10.65; and phenylalanine, 5.68 micromicromoles/A_{260}, for a total of 36. Phenylalanine is accepted by all three of these $tRNA_{coli}$'s from the *Neurospora* enzymes, and the amount was 11.36 micromicromoles/A_{260}. Thus less than one-third of the total available accepting sites were occupied by phenylalanine. A possible explanation of this phenomenon would be that only selected species of the isoaccepting forms of each aminoacyl tRNA were reactive and others were excluded by the *Neurospora* enzymes.

By chromatography on "Freon reverse-phase columns," $tRNA_{coli}$ was fractionated into three $tRNA^{ala}$'s and two $tRNA^{val}$'s. Testing these fractions with the *Neurospora* enzyme demonstrated that all three $tRNA^{ala}$'s and both $tRNA^{val}$'s accepted phenylalanine. The amount of phenylalanine accepted compared with either valine or alanine from *E. coli* enzymes ranged from 26 to 47% among these fractions. A variety of experiments were performed to demonstrate that the *Neurospora* enzyme was stable, was not contaminated with phenylalanine, and was not degrading the phe-$tRNA^{val}$ and phe-$tRNA^{ala}$ once they were formed. Thus we conclude that these fractions of $tRNA^{val}_{coli}$ and $tRNA^{ala}_{coli}$ are all partially reactive with this *Neurospora* phenylalanine-tRNA synthetase. The partial reactivity may be due to a number of factors: (1) the fractions may consist of more than one species of tRNA that were not resolved; (2) a given tRNA species may be able to exist in several forms indistinguishable by chromatography or the homologous enzyme but that do present distinguishable conformations to the heterologous enzyme. With regard to the latter possibility, an implicit assumption is that the alanine-tRNA synthetase of *E. coli* and the phenylalanine-tRNA synthetase of *Neurospora* recognize the $tRNA^{ala}$ according to different topographical features; in other words they do not utilize a common site on the tRNA molecule for "recognition." There have been suggestions that the anticodon is also employed as the enzyme recognition site, but this would clearly be inconsistent in the case of interaction of the *Neurospora* phe-tRNA synthetase with either $tRNA^{val}_{coli}$ or $tRNA^{ala}_{coli}$.

Biochemical Regulation

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Participation in Cooperative Programs:

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19.1 ENZYME TURNOVER IN VIVO: EFFECTS OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS

F. T. Kenney Joseph Kendrick
L. E. Roberson

Introduction. – By labeling rat liver tyrosine transaminase in vivo with ^{14}C-leucine, then measuring the rate of loss of radioactivity as labeled enzyme is replaced with unlabeled enzyme, we have determined the half-life of this enzyme to be 1.5 ± 0.3 hr.[1] Turnover of the bulk of the liver proteins proceeds with a half-life of about three days, so it is apparent that this transaminase is metabolically quite labile. This rapid turnover renders the enzyme level unusually sensitive to changes in the rates of enzyme synthesis and degradation, making this enzyme a useful model for the study of these processes. In the present study we are analyzing the effects of actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis, respectively, upon transaminase degradation. While much is known of the processes by which enzyme proteins are synthesized, very little has been done towards understanding the equally significant processes of enzyme degradation.

Results and Discussion. – Cycloheximide (100 μg/100 g) will block hormonal induction of tyrosine transaminase but has no effect on the basal level of enzyme over a 4- to 6-hr period. Since this interval encompasses several half-lives, the enzyme level would be expected to drop precipitously if synthesis were blocked and degradation continued at the normal rate. When the rate of enzyme synthesis was determined by pulse-labeling and immunological isolation, it was found that cycloheximide reduced synthesis virtually to zero over the entire 4-hr period tested. The conclusion

– that degradation as well as synthesis must be stopped by cycloheximide – was confirmed in experiments in which turnover of labeled enzyme was measured in rats treated with cycloheximide. The labeled enzyme was retained under these conditions, showing that degradation of this enzyme requires the synthesis of protein. Thus "degradation" is not simply the result of proteolytic activity, but represents a specific process which may involve an inactivation of the enzyme.

Inhibition of RNA synthesis by actinomycin, at a dose (100 μg/100 g) which will block hormonal induction, has caused variable effects on synthesis of the basal enzyme. In some experiments enzyme synthesis appears to be unaffected, which would indicate that synthesis occurs on stable templates. However, in others this drug markedly inhibits both synthesis and degradation. Interpretation of hormonal regulation of enzyme synthesis demands a knowledge of the stability of the RNA components involved, and we are currently trying to resolve these conflicting results.

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19.2 HORMONAL REPRESSION OF ENZYME SYNTHESIS IN "PATHOGEN-FREE" RATS

F. T. Kenney Joseph Kendrick
L. E. Roberson

Introduction. – We have experienced considerable variation in experimentation with the repression of hepatic enzyme synthesis by growth hormone, as discussed in a previous report.[1] Data now available (see Sect. 19.4, this report) indicate quite clearly that growth hormone does not act directly on the liver cells, but probably depends upon an initial extrahepatic effect which in turn causes repression of enzyme synthesis in the liver. An elucidation of the relationships involved here will undoubtedly be of great interest but is not pertinent to our primary aim, which is to analyze the mechanisms of repression. To do this requires a readily reproducible test system. Recently we gained access to rats from the Division's isolator breeding program. These animals are free of the common pathogens and exhibit none of the symptoms (e.g., respiratory distress) that we usually encounter in rats from commercial sources. They are being maintained in filtered cages in an isolated room until used in experiments.

Results and Discussion. – In the past, using rats from commercial channels, at least 25% of repression experiments were completely negative, and another 50% yielded so much variation that interpretation was impossible. These animals usually were found to harbor multiple infections when tested on entry into the Division. Prior to our experiments they must be either adrenalectomized or hypophysectomized, and these necessary manipulations undoubtedly contributed to the general malaise which they almost invariably exhibited during our experiments. In preliminary experiments with "pathogen-free" animals we have not yet encountered any difficulty in reproducibly eliciting repression of enzyme synthesis with growth hormone. The response is uniform, and it appears that the test system we require for analysis of mechanism is available in these animals.

These results imply that the response one sees to hormone administration can be profoundly influenced by the state of health of the animals used in experimentation. The difficulty becomes greater, of course, as knowledge and techniques become more precise and the parameters analyzed increase in complexity. Research in the area of hormone action is noted for inconsistency. When one considers the probable pathogen count of most animal colonies this is not particularly surprising.

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19.3 PREPARATION AND PROPERTIES OF FETAL RAT LIVER POLYRIBOSOMES

W. D. Wicks

Introduction. – Polyribosomes have been prepared from 20-day fetal rat liver and a few of their properties examined, especially the incorporation of [^{14}C]-valine and -phenylalanine.

Results and Discussion. – Homogenates of fetal liver were centrifuged at 30,000 × *g* for 30 min and the supernatant fraction applied to a 1.5 × 90 cm

Sephadex G-75 column. The column was developed with 0.02 *M* Tris pH 7.6 containing 0.01 *M* $Mg(OAc)_2$, 0.04 *M* NaCl, 0.10 *M* KCl, and 0.006 *M* β-mercaptoethanol at 3°. Fractions were collected and read at 260 and 280 mμ. A large amount of ultraviolet-absorbing material came off just after the void volume. This material was yellow colored and presumably contained all the polysomes, monosomes, activating enzymes, and other components involved in protein synthesis. The next peak had a low 260/280 ratio and all the fractions were red, indicating the presence of hemoglobin. A later peak exhibited a high 260/280 ratio and was colorless and represented transfer RNA.

The initial peak from the column was concentrated by the addition of dry Sephadex G-25 and centrifugation. The final clear yellow solution was used in all subsequent experiments.

Glycerol density gradients of this Sephadex fraction revealed several well-defined peaks of ribonucleoprotein material. Peaks corresponding to the presence of one, two, three, four, five, six, and seven ribosomal units were clearly separated. Components of higher molecular weight were present in somewhat smaller amounts and were not well resolved. The main polysome fraction generally contained five to six ribosomal units. At least three-fourths of the ribosomes of all preparations were in the form of polysomes. The addition of detergent had no effect on the polysome profile, suggesting that the bulk of fetal liver ribosomes were not bound to membranes in vivo. The RNA content of these preparations ranged from 10 to 20%.

The incorporation of [^{14}C]-valine and -phenylalanine by these preparations was quite vigorous even in the absence of added transfer RNA. Up to 400 micromicromoles of valine was incorporated per milligram of polysomal RNA during a 45-min incubation. Incorporation was absolutely dependent upon a supply of ATP and on the presence of Mg^{2+} and NH_4^+ ions. The omission of ^{12}C-amino acids removed the stimulatory effects of added transfer RNA, and the omission of GTP was partially inhibitory. Optimal $MgCl_2$ concentration was about 6×10^{-3} *M*. The amino-acid-incorporating activity of these polysome preparations was retained for at least three weeks when stored under liquid nitrogen.

Although the addition of small amounts of transfer RNA stimulated amino acid incorporation about twofold, larger amounts were inhibitory. These results may be explained on the basis that endogenous transfer RNA is bound to polysomes and is cycling somewhat more efficiently than added transfer RNA. Alternatively, there may be inhibitors in the transfer RNA preparations, although steps were taken to remove known inhibitors. The inhibitory effects of added transfer RNA were not due to any effects on aminoacyl-RNA formation.

[^{14}C]-Valyl-transfer RNA was not incorporated very readily, and the reaction was complete by 15 min, whereas it continued past 30 min when [^{14}C]-valine and transfer RNA were used.

The addition of poly U stimulated phenylalanine incorporation 10- to 20-fold, but poly UG had very little effect on the incorporation of valine.

Although the addition of fetal ribosomal RNA or ribosomal precursor RNA had little effect on valine incorporation, the addition of DNA-like RNA virtually abolished it. This effect was obtained only if the RNA was added during the first few minutes of incubation. The explanation of this phenomenon is presently unknown.

19.4 HORMONAL CONTROL OF ENZYME SYNTHESIS IN THE ISOLATED, PERFUSED RAT LIVER

C. B. Hager F. T. Kenney
G. R. Holloway

Introduction. – Previously described experiments from this laboratory have identified three nonadrenal, protein hormones which regulate the synthesis of hepatic tyrosine transaminase. Administration of pancreatic insulin or glucagon to adrenalectomized rats brought about a three- to fivefold induction of the enzyme.[1,2] Hypophyseal growth hormone was found to repress the synthesis of hepatic tyrosine transaminase.[3]

The question arises as to whether the response of tyrosine transaminase to these nonadrenal hormones occurs as a result of their direct influence on hepatic cells. This problem is currently being investigated in the isolated, perfused rat liver.

Results and Discussion. – The rat liver perfusion technique of Miller *et al.*[4,5] is being used, employing livers from adrenalectomized rats. The perfusion medium consists of heparinized whole rat blood from adrenalectomized animals with an equal volume of Krebs-Ringer bicarbonate buffer solution containing added glucose, casein hydrolysate, L-tryptophan, penicillin, and streptomycin.

With such a system the level of tyrosine transaminase remains fairly constant for several hours. Hydrocortisone (1.5 to 2.0 mg per 100 g body weight of the liver donor) elicits a two- to threefold increase in the activity of the enzyme when added to the perfusion medium at the start of the experiment and infused into the medium at a constant rate. This level of induction corresponds to that reported previously in the isolated, perfused rat liver.[6,7] Following a lag period of 1 to 2 hr, enzyme synthesis increases linearly for the next 2 or 3 hr.

Insulin and glucagon are also found to stimulate tyrosine transaminase activity in the isolated, perfused rat liver. Consequently, this response can be assumed to result from a direct action in the liver of these hormones. Both the insulin and glucagon effect can be observed 1 hr after addition of either hormone to the perfusion medium. Insulin is as effective as hydrocortisone at dosage levels corresponding to those employed in the in vivo studies. However, preliminary investigations using glucagon imply a lesser effect of this hormone in the isolated, perfused rat liver than observed when this hormone is administered in vivo. Also, while the enzyme level begins to return to normal about 3 hr after insulin or glucagon treatment in vivo the level continues to increase linearly throughout the duration of the perfusion in the isolated rat liver (4 to 6 hr). Since these hormones are constantly infused into the perfusion medium, these data indicate that the decrease in enzyme level following hormonal induction in vivo occurs as the result of the catabolism of the hormones.

Unlike the direct effects of insulin and glucagon on tyrosine transaminase activity noted in the isolated, perfused rat liver, repression by growth hormone is not observed. The question now arises as to how growth hormone, when administered to the rat in vivo, brings about repression of this enzyme. Further experiments are now in progress which we hope will elucidate this problem.

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19.5 RNA SYNTHESIS IN RAT LIVER UNDER THE ACTION OF INSULIN

Claudio Pellegrino F. T. Kenney

Introduction. – It was previously shown in this laboratory that hydrocortisone, insulin, and glucagon are all able to increase the hepatic levels of tyrosine transaminase in rats that were adrenalectomized or hypophysectomized or both.[1] Pulse labeling of the enzyme by ^{14}C-amino acids and its isolation by precipitation with specific antiserum showed that this effect, in the case of each of these three hormones, was due to a stimulation (three- to sevenfold) of the rate of synthesis of this enzyme.

Actinomycin inhibits tyrosine transaminase induction by hormones, hence the conclusion that transcriptional events must be involved in this process. In fact, hepatic RNA synthesis is increased by hydrocortisone,[2,3] insulin,[4] and growth hormone.[5,6]

It is thus clear that hormones of different chemical structure (two proteins and a steroid) give rise to similar effects on rat liver. In an effort to analyze further their mechanism of action, a study of the modifications of RNA synthesis induced by insulin has been undertaken.

Results and Discussion. – The results so far obtained are preliminary and seem to indicate that a fraction of microsomal RNA, salt-precipitable, with a sedimentation constant of about 4 s, shows a higher specific activity 2 hr after insulin administration. Analysis of this and other RNA fractions with various techniques is planned.

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Microbiology

Electron Transport Mechanisms in Microorganisms

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[a]Dual assignments

20.1 KINETICS OF MALIC-LACTIC TRANSHYDROGENASE

M. I. Dolin

Interest in malic-lactic transhydrogenase of *Micrococcus lactilyticus* stems from considerations that have been summarized previously.[1] In order to obtain some insight into the mechanism of the reaction, the steady-state kinetics of the transhydrogenation have been under investigation.

The enzyme can be prepared in a form in which 90% of the total NAD is in the form NADH. This firmly bound NADH is 20 times as fluorescent as free NADH. On the addition of pyruvate, a stable nonfluorescent complex is formed from which lactate is not liberated until malate is added. The steady-state kinetics of the reaction in the direction of lactate formation require the formation of abortive complexes between reduced enzyme (E·NADH) and malate and between the nonfluorescent complex and an additional molecule of pyruvate. Initial steady-state kinetics are described by the following equation:

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{A}\left(1 + \frac{B}{K_{ib}}\right) + \frac{\phi_2}{B}\left(1 + \frac{A}{K_{ia}}\right),$$

in which A = pyruvate, B = malate, and K_{ia} and K_{ib} are respectively the dissociation constants for the abortive complexes formed with pyruvate and malate. The coefficients ϕ_0, ϕ_1, and ϕ_2 are composed of rate constants and are expressed in the notation of Dalziel.[2] Equations of this form can be derived for several mechanisms involving binary or ternary complexes of enzyme and substrates. All the parameters have been evaluated, and it has been shown that the theoretical rate equation matches the experimental data over a 100-fold range of malate and pyruvate concentration.

The coefficient ϕ_1 is the reciprocal of the second-order rate constant for the reaction of enzyme with pyruvate. Both steady-state and stopped-flow kinetics give the same value for this constant ($\sim 8 \times 10^4\ M^{-1}\ \mathrm{sec}^{-1}$). The other coefficients, ϕ_0 and ϕ_2, may be composed of single rate constants or combinations of constants, depending on the exact mechanism of transhydrogenation. More precise definition of the reaction mechanism will require a study of the kinetics in the direction of malate formation. As a preliminary step, it was necessary to define the conditions under which the rate of enolization of oxalacetate does not limit the rate of oxalacetate utilization. Computer solutions have been obtained[3] which should aid in the design of these experiments.

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Biophysics

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Excited-State Biophysics

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Radiation Biophysics

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Molecular Biophysics

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Participation in Cooperative Programs:

AEC-NIH Chemical Carcinogenesis (22.12–22.14)

[a]Dual assignments
[b]Biology Division postdoctoral investigator
[c]Student trainee
[d]Leave of absence to Hammersmith Hospital, London, England, under sponsorship of National Science Foundation Senior Postdoctoral Fellowship
[e]Research participant
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21.1 ROLE OF THE λ REPRESSOR

R. A. Weisberg

Temperature shift experiments using lysogens of a mutant of bacteriophage λ (λ857) whose repressor (coded for by the cI gene of the phage) is inactivated at high temperature and becomes active again when the temperature is lowered show that (*a*) detachment of the prophage from the bacterial chromosome occurs independently of and earlier than commitment of the prophage to vegetative multiplication (and concurrent commitment to cell death); (*b*) detachment of the prophage requires protein synthesis while the phage repressor is inactivated; and (c) commitment to vegetative multiplication and cell death requires thymine while the repressor is inactivated. These experiments demonstrate the existence of two repressor-sensitive functions in phage λ. The first is the synthesis of a protein(s) necessary for prophage detachment; the second is a thymine-requiring function – presumably the initiation of phage DNA synthesis – which, when "completed," enables the phage to multiply and kill the cell in the presence of active repressor.

Two other λ mutants with temperature-sensitive repressors have been studied. The first (λU32) –

believed on the basis of other evidence to synthesize a repressor which is irreversibly inactivated by heat – is induced to multiply even when protein synthesis is blocked or thymine is withheld at high temperature. In addition, it is not possible in lysogens of this phage to separate prophage detachment from vegetative multiplication by the appropriate temperature-shift experiments. These findings are consistent with the idea of irreversible heat inactivation of the repressor in this mutant.

The second mutant (λt1) is believed to synthesize a repressor which is inactivated at high temperature, but which is only partially reactivated when the temperature is lowered. Our experiments show that: (*a*) prophage detachment can be dissociated from commitment to vegetative multiplication by the appropriate temperature-shift experiments; (*b*) protein synthesis at high temperature is required for prophage detachment to occur; and (*c*) thymine is *not* required at high temperature for commitment to vegetative multiplication to occur. These findings, while not inconsistent with the idea of a partially reversible heat inactivation of the repressor in this mutant, are not as yet interpretable in a simple and unambiguous way.

21.2 STUDIES OF PLASTID RIBOSOMES

Ann B. Jacobson Peggy P. Hester

Introduction. – Our previous report[1] suggests that ribosomal RNA from proplastids of etiolated corn leaves may contain at least one component which sediments differently than the RNA of nonplastid cytoplasmic ribosomes. The evidence for this statement was derived from correlated studies combining electron microscope autoradiography with biochemical isolation procedures. It was shown that under conditions where cells from etiolated leaves synthesize primarily plastid RNA, the sedimentation rate of the heavy ribosome component from plastids was slightly slower than the same component from cytoplasmic ribosomes. This argument was indirect, and it remained necessary to confirm these observations with RNA from pure proplastid preparations.

Methods and Results. – In recent months it has proved possible to obtain relatively clean plastid preparations. The major contaminant in the preparations has been bacteria which grow on the leaf surfaces of the plants themselves. In order to eliminate these bacteria completely it appears necessary to grow the plants in a sterile environment. However, careful differential centrifugation has permitted us to reduce the level of bacterial contamination to approximately 0.0001%. It has also been possible to improve the yield of proplastid per gram fresh weight of leaves at least 50-fold over our previous values. Thus 200 g of etiolated corn leaves currently yields 3×10^9 very clean proplastids, or 1.2 mg of proplastid RNA.

Figure 21.2.1 presents some data obtained with clean proplastid RNA. The top curve (*A*) shows a sucrose gradient (17 to 34%) run for 48 hr with whole-leaf RNA. The component peaks have been labeled I, II, III, IV, and V. Both II and IV are not normally seen in RNA from other tissues. DNAse removes only component IV. The middle curve (*B*) shows plastid RNA run on a similar gradient. The bottom curve (*C*) was obtained by placing equal amounts of whole-leaf and plastid RNA on the same gradient. Clearly, peak II is the heavy component of plastid ribosomal RNA, and it sediments more slowly than the RNA (I) of the nonplastid ribosomes.

Recently, a similar observation has been made with ribosomes from mature chloroplasts in another laboratory.[2] The authors report sedimentation values of 23*S* and 16*S* for ribosomal RNA from chloroplasts and 25*S* and 16*S* for cytoplasmic ribosomes. The chloroplast values are identical with values obtained for bacterial RNA. It is not clear yet whether our RNA will give these same values or not.

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21.3 SATELLITE DNA'S OF CRUSTACEA

Dorothy M. Skinner L. L. Triplett

Ten to thirty percent of the DNA of certain Crustacea (various species of the genus *Cancer*) is a satellite which can be separated by its buoyant density in CsCl from the so-called main band DNA. Whereas this satellite is composed of 97% adenine and thymine, the rest of the DNA is composed of the four bases in amounts more nearly equal to each

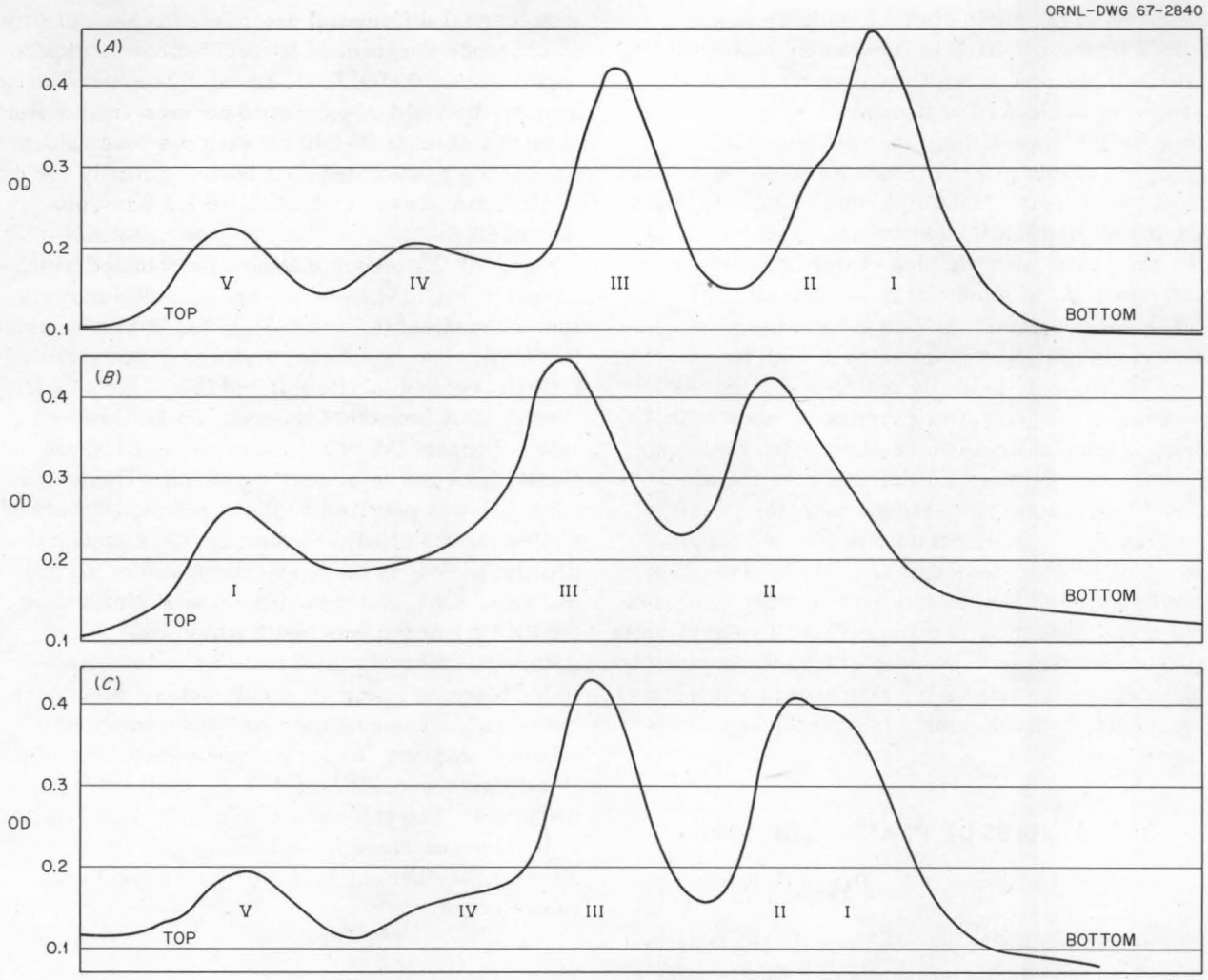

Fig. 21.2.1. Comparison of Sedimentation Rate of Proplastid and Cytoplasmic Ribosomal RNA. (*A*) Whole leaf RNA, (*B*) proplastid RNA, (*C*) mixture of equal amounts of proplastid and cytoplasmic RNA.

other.[1] Many of the chemical and physical properties of the adenine-thymine-rich satellite have been studied by other workers; however, nothing is known of its localization in the cell, mode of synthesis, or its biological role, if any. We have found the AT satellite DNA in the tissues of another crustacean, the land crab *Gecarcinus lateralis*. In addition, we find another DNA satellite, high in GC, in the tissues of *G. lateralis* and also in DNA isolated from the testes of one of the species of *Cancer* (*C. pagurus*) previously studied. The existence of the poly AT was thought to be characteristic of the genus *Cancer*, having been found previously in eight species of that genus. It is absent from *Carcinus*, which is another crab in the cancroid group of the subsection Brachygnatha. *Gecarcinus*, which contains the AT satellite, and *Maia squinado*, which does not, are grapsoids, members of the other major group of the Brachygnatha. Thus it is evident that the distribution of this particular satellite does not coincide with the classification of these animals on a taxonomic basis, as was once suggested.

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21.4 UV-IRRADIATION STUDIES OF NATURALLY OCCURRING "POLY d(A-T)"

Dorothy M. Skinner R. B. Setlow

A naturally occurring DNA with physical characteristics similar or identical to those of alternating d(A-T) has been isolated from the crab *Gecarcinus lateralis*. If the purified "d(A-T)" is irradiated with ultraviolet at wavelength of 280 mμ, there is a decrease in the absorption at ~270 mμ. This decrease can be reversed by subsequent irradiation with 239 mμ. This behavior is characteristic of polynucleotides containing adjacent pyrimidines, as they form cyclobutane dimers. Furthermore, the extent of the spectral change, as well as the rates of change with dose, indicates that thymine-thymine dimers are being formed. It appears that the naturally occurring polymer contains at least 2% adjacent thymines and that the remainder of the molecule is probably alternating adenine and thymine residues.

21.5 INTERRUPTIONS IN THE CHROMOSOMAL DNA OF A SLIME MOLD

R. A. McGrath R. W. Williams

Centrifugation in alkaline sucrose gradients[1] has been used to determine the average size of single-stranded pieces of DNA in synchronously dividing cultures of the slime mold *Physarum polycephalum*. We calculate from this size the fewest number of pieces of DNA into which the average chromosome dissociates under alkaline conditions.

Methods. – Synchronous cultures of slime mold were grown in the presence of ^{3}H-thymidine for one or more cycles of division and DNA synthesis in order to label DNA. Nuclei were isolated at interphase (determined by microscopic examination of samples from the culture) by a modification of Mohberg's technique. Approximately 2×10^6 nuclei in a volume of 20 to 50 μl were pipetted into a layer of 0.5 *N* NaOH that was floating on top of a 5 to 20% alkaline (pH 12.0) sucrose gradient. Preparations were allowed to remain at room temperature for 1 hr in order to disrupt nuclei and denature the DNA before centrifugation at 30,000 rpm for 2.5 hr at 20°C in an SW-39 rotor of a Spinco model L centrifuge. After centrifugation the tubes were punctured and two-drop fractions were collected on filter paper disks. Disks were processed to remove acid-soluble radioactive material and were counted in a liquid scintillation counter.

Results. – Figure 21.5.1 shows that single-stranded DNA obtained from slime mold nuclei by this procedure has a sedimentation constant ($S_{20,w}$) of about 59. Since our handling methods allow detection of pieces of DNA as large as 2 to 4×10^8 daltons, it is clear that the small pieces isolated from slime mold do not result from uncontrolled breakage or shearing of larger pieces. Since there is no difference in the sedimentation constant of DNA from slime mold whether incubated prior to centrifugation for 1 or 16 hr in 0.5 *N* NaOH, the small size of the pieces of DNA cannot result from alkaline degradation of larger pieces. To test whether nucleases had degraded DNA during the nuclear isolation procedure, labeled cultures were frozen in liquid nitrogen, ground to a fine powder, and this powder was sprinkled directly on top of a layer of 0.5 *N* NaOH floating on top of the alkaline sucrose gradient. Subsequent centrifugation and analysis of data showed that the sedimentation constant of DNA isolated in this manner was not larger than that obtained from isolated nuclei. These results show that nuclear DNA is not reduced in molecular weight

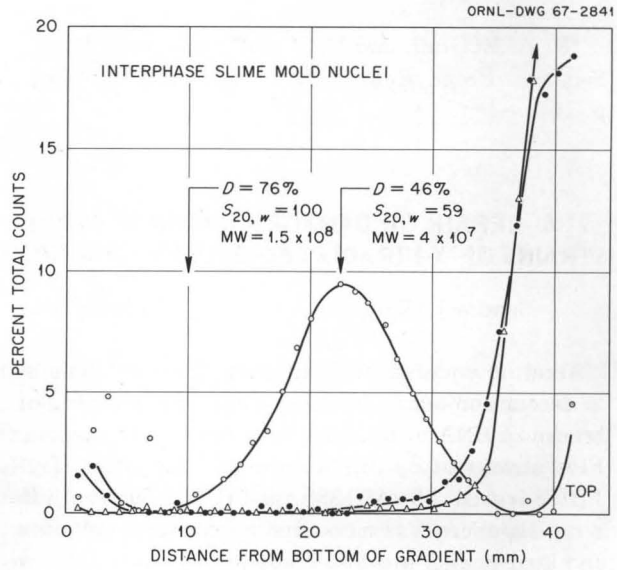

Fig. 21.5.1. Sedimentation Profile of Slime Mold Nuclei in Alkaline Sucrose Gradients. ○, labeled with ^{3}H thymidine; ●, labeled with ^{3}H uridine; Δ, labeled with ^{14}C amino acid mixture. D = distance from top of gradient; $S_{20,w}$ = sedimentation constant; MW = molecular weight in daltons.

during the nuclear isolation procedure. When cultures of slime mold were labeled with RNA or protein precursors and then subjected to alkaline sucrose gradient centrifugation, 95 and 99%, respectively, of the radioactivity was found in regions of the gradient other than that in which DNA sediments (see Fig. 21.5.1). If the DNA were contaminated with either RNA or protein this should lead to an impression of higher sedimentation constant, that is, fewer pieces of DNA than that obtained.

On the basis of an average chromosome number of 50 per nucleus and a nuclear content of 6×10^{11} daltons of DNA (a sedimentation constant of 59 is approximately equal to a molecular weight of 4×10^{7} daltons), we are led to conclude that each chromosome dissociates into (or contains) about 300 single-stranded pieces of DNA. It is possible that the intact chromosome is held together by alkaline labile linkers that break down under our experimental conditions, or that true gaps or interruptions exist between adjacent pieces of DNA. Alternatively, the presence of breaks or interruptions at any time may reflect maintenance (i.e., correction of everyday wear and tear) of the DNA.

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21.6 REPAIR OF DAMAGED DNA IN SEVERAL STRAINS OF X-IRRADIATED *ESCHERICHIA COLI*

Sandra J. Roland R. A. McGrath

Alkaline sucrose gradient centrifugation[1] was used to detect and assay both breakage and rejoining of breaks in DNA of bacteria following x-ray exposure. Five strains of *E. coli* were used: WP_2 Hcr^- Try^-, AB1884, AB1885, AB1886, and B_{s-3}. Survival after x-ray exposure was measured by counting colonies on nutrient agar plates. Labeling and radiation exposure conditions, as well as the assay technique following centrifugation in alkaline sucrose, have been described elsewhere.[1] A comparison of nonirradiated, irradiated-unincubated, and irradiated-incubated (in growth medium after x-ray exposure) cells were made. Immediately after exposure to 20 kr of x rays the molecular weight of pieces of DNA in all strains studied was reduced by a factor of 2. During a subsequent 40-min incubation period in growth medium all strains other than B_{s-3} showed evidence of break-rejoining, that is, the molecular weight of DNA reduced by x-ray exposure was observed to increase. Increases between 23 and 40% occurred. By comparison, it was previously shown that strain B/r increased by about 100% and B_{s-1} showed, like B_{s-3}, no increase.[1] A correlation exists between survival after exposure and the capacity of particular strains to repair damaged DNA.

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21.7 ENZYMES INVOLVED IN DIMER EXCISION IN VITRO

W. L. Carrier Janet R. McGrath
R. B. Setlow

Extracts of *Micrococcus lysodeikticus* are able to excise pyrimidine dimers from uv-irradiated DNA.[1] Excision is measured by the ability of the extract to remove dimers from the acid-insoluble fraction of uv-irradiated DNA labeled with ^{3}H-thymidine. Dimers excised from the DNA appear as parts of acid-soluble oligonucleotides.

Chromatography of a streptomycin-treated fraction of the extract on DEAE-cellulose has yielded two fractions necessary for the excision of dimers in vitro. The first component involved in the excision process does not stick to DEAE-cellulose. It is an endonuclease which shows a specificity for uv-irradiated DNA. We assay for this nuclease by its ability to make starting points in uv-irradiated DNA for the subsequent action of highly purified venom phosphodiesterase.

The second component needed to render dimers acid-soluble elutes from DEAE-cellulose with 0.5 *M* phosphate buffer, pH 7.5. This fraction also contains DNA polymerase activity. We have not detected in it any nuclease activity that is specific for uv-irradiated DNA. When irradiated DNA is allowed to react with the uv-specific endonuclease, then heated at 62°C for 10 min, the addition of the second component results in the excision of dimers. Treatment of irradiated DNA with the second component fol-

lowed by the uv-specific endonuclease does not result in dimer excision. Thus, as expected, the first step in the excision of dimers in this system is the action of an endonuclease specific for uv-irradiated DNA.

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21.8 CHAIN BREAKS AND CROSS LINKS IN DNA IRRADIATED IN VIVO WITH ULTRAVIOLET LIGHT

R. B. Setlow R. W. Williams

Many experiments on DNA irradiated in vitro have shown that uv radiation causes chain breaks and cross links. Similar results were observed for the DNA of irradiated T7 phage.[1] We have observed chain breaks and cross links in bacterial DNA irradiated in vivo.

Methods and Results. – *E. coli* B_{s-1} (a nonrepairing strain) was labeled with ^{3}H-thymidine and irradiated with 265-mμ radiation. The cells were lysed and centrifuged in alkaline sucrose to determine the distribution of single-stranded DNA among the various molecular weight classes.[2] The number-average molecular weight decreased as the radiation dose increased, and the distribution of molecular weights contained pieces both smaller and larger than found in unirradiated bacteria. The data are consistent with the notion that uv radiation induces one alkali-stable cross link for each two alkali-labile chain alterations (breaks). There are tenfold fewer breaks than would have been predicted from the data on bacteriophage. A dose of 200 ergs/mm^2 makes on the average 1000 pyrimidine dimers but only one break and one-half a cross link per bacterial chromosome.

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21.9 EXCISION OF PYRIMIDINE DIMERS IN *ESCHERICHIA COLI*

R. B. Setlow W. L. Carrier

Introduction. – Dimers between adjacent pyrimidines in DNA act as lesions in bacteria, and radiation-resistant strains recover from the effects of dimers by processes that include the excision of dimers from DNA. Sensitive strains – in particular Hcr$^-$ ones – do not excise when tested at the same doses as used for resistant cells. We report data that indicate (*a*) that excision of dimers in resistant cells is not associated with the growing point of the bacterial chromosome and (*b*) that sensitive cells do not excise dimers even at doses low enough for appreciable numbers of cells to survive.

Methods and Results. – *Excision and Growing Points.* – Cells of *E. coli* 15T$^-$ were fully labeled with ^{14}C-thymidine, then given a 5-min pulse of ^{3}H-thymidine to label the chromosome region near the growing point, transferred to nonradioactive medium, and immediately irradiated with 200 ergs/mm^2 of 265 mμ to make ~1000 dimers per chromosome. Cells were incubated for various periods of time and the acid-insoluble fractions analyzed for dimers labeled with ^{3}H and ^{14}C. The results in Fig. 21.9.1 show that excision is rapid and, within ±5%, is the same

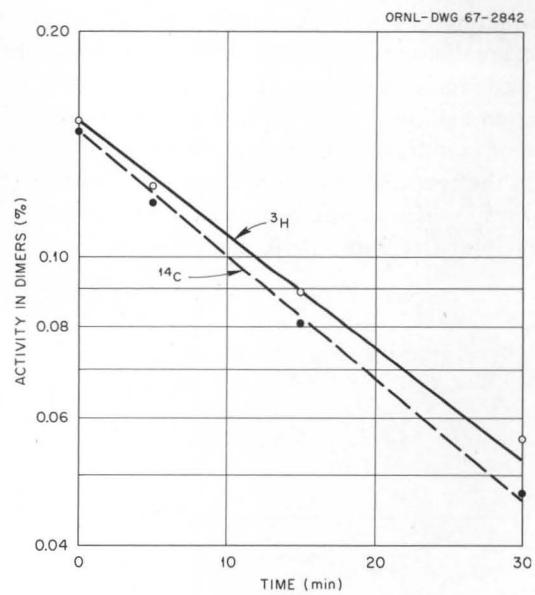

Fig. 21.9.1. The Radioactivity (%) in Dimers in the Acid-Insoluble Fractions of *E. coli* 15T$^-$ at Several Times After UV Irradiation. ^{14}C represents the fully labeled and ^{3}H the region behind the growing point of the chromosome. 5 min pulse, 15T$^-$, 200 ergs/mm^2, average of 2.

for the newly synthesized region and the average DNA. A selective excision mechanism does not act in the region of the growing point of the chromosome.

Excision in Sensitive Strains. – Several Hcr$^-$ strains were labeled with ^{3}H-thymidine and irradiated with doses that make 100 to 150 dimers per chromosome. The acid-insoluble fractions were analyzed for dimers at 0 and 60 min after irradiation. At the latter time DNA synthesis had resumed in the irradiated culture. The results are given in Table 21.9.1. Even at doses in the "biological range" for these sensitive strains there is little, if any, excision. The rate of dimer excision is $\leq$1% of that observed in Hcr$^+$ strains (compare Table 21.9.1 and Fig. 21.9.1). It is important to recognize that most Hcr$^-$ strains survive to form colonies and resume DNA synthesis even though many dimers remain in their chromosomes.

21.10 DIRECT DEMONSTRATION THAT YEAST PHOTOREACTIVATING ENZYME AND LIGHT MONOMERIZE THYMINE-CONTAINING DIMERS IN UV-IRRADIATED DNA

J. S. Cook

Although it is generally accepted that the repair of uv-irradiated DNA by photoreactivating enzyme and light involves the reversion of cyclobutane-containing pyrimidine dimers to pyrimidine monomers, the reaction has been directly demonstrated only for the case of cytosine-containing dimers.[1] In the present work, the reaction is quantitatively demonstrated for the thymine-containing dimers in *E. coli* DNA. Ultraviolet-irradiated DNA labeled with ^{3}H-thymine was mixed with unirradiated DNA labeled with ^{14}C-thymine; the mixture was exposed to light in the presence of yeast photoreactivating enzyme, then hydrolyzed and chromatographed. As photoreactivation progresses, the loss of ^{3}H activity in the dimer region of the chromatogram is accompanied by the stoichiometric increase in the ^{3}H/^{14}C ratio in the thymine region. In order to demonstrate this reversion of dimers to monomers, it is necessary to allow for a small but real chromatographic separation of ^{3}H- and ^{14}C-labeled thymine.

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21.11 DISTRIBUTION OF PHOTOREACTIVATING ENZYMES

J. S. Cook Janet R. McGrath

The existence of photoreactivating enzyme in a tissue may be inferred from (1) the ability of an extract of the tissue in the presence of light to reverse the biological effects of ultraviolet irradiation on transforming DNA and (2) the loss of this ability after the extract has been heated to 65°C for 10 min. Other criteria (nondialyzability of the active component of the extract; ability of the extract in the presence of light to split pyrimidine dimers formed in DNA by ultraviolet irradiation) may be and have been used to demonstrate the existence of the photoreactivating enzyme in a particular tissue.

Table 21.9.1. Radioactivity in Dimers (Percentage of Total) in the Acid-Insoluble Fraction of the UV-Irradiated Strains of *Escherichia coli*

Strain	Time After UV (20 to 30 ergs/mm^2, 265 mμ)	
	0 min	60 min
WP2 hcr$^-$	0.018	0.018
B_{s-8}	0.018	0.024
B_{s-1}	0.022	0.021
AB1884	0.023	0.021
B_{s-3}	0.015	0.013

Since the enzyme is found in some tissues which are never exposed to light (e.g., sea urchin testis; the sperm, which are exposed to light on being extruded into seawater, do not contain the enzyme), it is possible that the enzyme serves some function other than photoreactivation. As a preliminary to investigating this possibility, a survey has been made of the distribution of the enzyme.

Phylogenetically, the enzyme has been found in echinodermata, crustacea, insects, fish, amphibia, and bird (chick) embryo. The enzyme has not been found in Tetrahymena, adult chick, nor, despite an intensive search under a wide variety of conditions, in mammals. The tissue distribution is also broad; in animals where activity is found at all, it is found in virtually every tissue except blood serum.

The cellular distribution is under investigation. In frog liver the enzyme appears to be in the soluble fraction. It is readily separable from mitochondria and mitochondrial enzyme activity and from nuclei and DNA. Whether the dissociation of the enzyme activity from the nuclei is an artifact of preparation is not yet certain. There is physiological evidence in other cellular systems (particularly sea urchin eggs) that the enzyme is active in the cytoplasm as well as the nucleus; that the enzyme is apparently in the soluble fraction is thus not contradictory to physiological data.

21.12 DELAYED RESPONSE IN THE PHOTOREACTIVATION OF TRYPTOPHANASE INDUCIBILITY IN *ESCHERICHIA COLI* B

P. A. Swenson

Ultraviolet radiations inhibit the formation of induced enzymes in *Escherichia coli* B. Three induced enzymes, β-galactosidase, tryptophanase, and D-serine deaminase, are about equally sensitive to 2537-A radiation in both a radiation-resistant strain (B/r) and in a radiation-sensitive strain (B_{s-1}). The three enzymes differ, however, in their abilities to be photoreactivated. The criterion for photoreactivation (P. R.) is an increase in enzyme-forming ability above that for cells not receiving the P. R. treatment. Ultraviolet doses of about 400 ergs/mm^2 to B/r inhibit the formation of the three enzymes by 90 to 95%. Following P. R. treatment at room temperature, immediate induction, and incubation at 37°C, β-galactosidase shows the most P. R. ($2\frac{1}{2}$ times increase). D-Serine deaminase shows about one-third the photoreactivability of β-galactosidase, but tryptophanase shows no photoreactivability. Tryptophanase, however, shows a delayed P. R. effect. If, following uv and P. R. treatment, B/r cells are preincubated at 37°C for 10, 20, or 30 min before induction, a graded amount of P. R. is obtained. When cells are induced immediately after P. R., a small amount of P. R. occurs after about 60 min. No immediate P. R. is obtained when P. R. treatment is carried out at 37°C.

When B/r and B_{s-1} cells are irradiated at lower doses, ~120 ergs/mm^2, immediate photoreactivation of tryptophanase is obtained if P. R. treatment is given at 37°C but not if given at room temperature. Delayed P. R. is observed in B/r under the latter conditions, 30 min after addition of inducer. As is the case with B/r at high doses, delayed P. R. is also obtained when either strain receives P. R. treatment at room temperature and a preincubation period of 30 min is allowed before addition of inducer.

The results suggest that temperature-dependent steps following the photochemical events in the P. R. of the tryptophanase gene are necessary for the expression of this gene.

21.13 EFFECTS OF ULTRAVIOLET IRRADIATION ON RESPIRATION OF RADIATION-RESISTANT AND SENSITIVE STRAINS OF *ESCHERICHIA COLI*

Barbara A. Hamkalo P. A. Swenson

The effect of uv irradiation on the respiration of strains of *E. coli* which differ in radiation sensitivity, as determined by survival, is being studied by Warburg manometry. Unirradiated log-phase cells of B/r and B_{s-1} show an exponential increase in oxygen consumption with time. After a heavy dose of uv (~400 ergs/mm^2), both strains respire at the same rate as the controls for 1 hr. After this time oxygen consumption in B/r, the radiation-resistant mutant, ceases, whereas the sensitive mutant, B_{s-1}, continues to respire at a linear rate.

If cells are irradiated in stationary phase, there is no apparent effect of the same dose of uv on oxygen consumption. This result rules out a direct effect of uv on the respiratory enzymes. Minicells[1] which are derived from a K12 parent strain of *E. coli* and which possess no DNA[1] also show no effect of uv on respiration. The uv-damaged respiratory activity in log-phase cells can be photoreactivated. Since log-phase

cells which are synthesizing DNA are more uv sensitive than stationary cells, the uv effect on respiration may be a reflection of damage to a process dependent on DNA synthesis.

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21.14 ACTION SPECTRA FOR SURVIVAL AND PHOTOPRODUCT PRODUCTION IN *B. MEGATERIUM* SPORES

J. E. Donnellan, Jr. J. L. Otis
R. S. Stafford

Introduction. – The photoproducts formed by ultraviolet irradiation of bacterial spores have been postulated to be involved in the mechanism leading to spore death.[1] It should follow, therefore, that colony formation and photoproduct production should share the same functional dependence on the wavelength of irradiation. We have determined the action spectra for these two events.

Results and Discussion. – Spores of *B. megaterium* labeled with tritiated thymidine were irradiated with light of various wavelengths and survival curves and rates of production of photoproducts determined. It was difficult to obtain reproducible results until we recognized that the age of the spores and the conditions under which they are produced can affect both survival and photoproduct production. The most accurate data have been obtained at wavelengths of 2378, 2652, and 2804 A. At these wavelengths there is no difference in the survival curves or in the rate of photoproduct production. They are all equally effective. Other less precise data suggest that minima occur in the action spectra for both events at 2483 A and that the action spectra decrease at 2894 A.

From these data we conclude that colony formation and photoproduct production share the same functional dependence on the wavelength of uv irradiation. This result strengthens the hypothesis that spore photoproducts are involved in spore death.

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21.15 THE ULTRAVIOLET PHOTOCHEMISTRY OF GERMINATING SPORES

R. S. Stafford J. E. Donnellan, Jr.

Introduction. – Ultraviolet irradiation of bacterial spores produces thymine-containing photoproducts different from the thymine photoproducts produced in vegetative cells.[1] The spore photoproducts are involved in the mechanism leading to spore death.[2] Stuy and Irie *et al.* have shown that germinating spores go through a stage in which they become immune to uv irradiation as exhibited by tailing of survival curves.[3] We have confirmed their findings and have studied the production of thymine photoproducts in spore DNA during germination.

Methods. – Spores of *B. subtilis* strain W168 ind$^-$, thy$^-$ were placed in growth medium, and, after various times, aliquots were removed for studies of uv survival or photoproduct production. For the latter studies spores were produced in medium containing tritiated thymidine. In some experiments spores of *B. megaterium* strain QMB1551 were used. Photoproducts were isolated by paper chromatography and measured in a liquid scintillation counter.

Results and Discussion. – Survival curves of germinating *B. subtilis* exhibited tails which were, within experimental error, unaffected by further uv exposure. This type of survival curve is observed with many organisms at very low survival. With germinating spores, however, the level of survival at which the survival is independent of dose is a function of the germination time of the spores. It goes through a maximum of 7 to 10% survival after 10 min. This is best shown by plotting the level of survival for a single large dose of irradiation vs the time of germination. Similar single-dose experiments were performed using *B. megaterium* spores. Spores of this organism germinate very rapidly, the process being complete after about 3 min. It would be expected that during long irradiations all spores in the population would enter or leave the resistant state during the period of irradiation. It should be possible, however, to find an irradiation time during which some spores neither enter nor leave the resistant state, that is, the time during which the spore remains in that state. This time should yield the highest survival in the single-dose experiments. We have irradiated germinating *B. megaterium* spores with 4000 ergs/mm^2 for exposure times of 150, 15, and 5 sec and find maximum survivals of 7, 25, and 32% respectively. It is possible that shorter exposures may yield slightly higher maximum survivals.

The production of photoproducts in germinating spores of *B. subtilis* was studied. After various periods of germination labeled spores were irradiated with a single dose and the amounts of spore photoproducts or thymine dimers measured. As a function of germination time the production of spore photoproduct decreases after a very brief lag. The production of thymine dimer, however, is delayed for a longer period of time. These results suggest that the DNA of bacilli is found in three states. One state is that found in bacterial spores. In this state thymine dimers are not formed but spore photoproducts are. A second state is that of vegetative cells or DNA in solution in which normal thymine dimers are produced. The third state is the very transient one found in germinating spores. In this state no thymine photoproducts are produced and the cells are very resistant to ultraviolet radiation.

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21.16 THE BIOLOGICAL SIGNIFICANCE OF A NEW PHOTOPRODUCT FORMED IN DNA AT 77°K

R. O. Rahn Jane K. Setlow[1]

Introduction. – A thymine-containing photoproduct found in DNA irradiated at 77°K has many properties similar to those of a photoproduct found in irradiated bacterial spores[2] and is probably the same compound. The spore photoproduct is not repaired by photoreactivating enzyme from yeast. The nonphotoreactivability of the photoproduct has been used to obtain evidence for its lethal effect on transforming DNA.

Methods and Results. – The loss of transforming ability of *Hemophilus influenzae* DNA was measured as a function of dose at 298 and 77°K at two ultraviolet wavelengths with and without maximum photoreactivation, and chromatographic analysis was made of the photoproducts. After 298°K irradiation at 2800 A or at 2380 A, more than 80% of the damage is photoreactivable with yeast enzyme (and the thymine-containing dimers, known to be a substrate for the photoreactivating enzyme, are the main photoproduct). However, following 2800-A irradiation at 77°K, where the dimer and the spore-type photoproduct appear in equivalent amounts, less than 60% of the damage is photoreactivable. At 2380 A, the photoreactivable sector is less than 50% for the low-temperature irradiation, in accordance with the proportionally larger amount of spore-type photoproduct formed under these conditions.

Conclusions. – The new photoproduct formed in DNA at 77°K inhibits transformation.

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21.17 NATURE OF PHOTOPRODUCT FORMED IN DNA AT 77°K

R. O. Rahn J. L. Hosszu

It was established previously that thymine dimer formation is severely retarded when DNA is irradiated at 77°K. However, evidence has been obtained that a new thymine photoproduct is formed at 77°K. This new photoproduct is of interest because of its similarity with the thymine photoproduct found in irradiated spores. The following features have been identified for the new photoproduct obtained when DNA is dissolved in a 50% ethylene glycol mixture and irradiated with 2800 A at 77°K.

1. $R_F = 0.4$ for butanol/water/acetic acid solvent with phosphate buffer present,
2. stable to formic acid hydrolysis, 170°C,
3. nonreversible with short-wavelength uv,
4. competes with thymine dimer for adjacent thymine moieties,
5. growth rate parallels that of thymine dimer, both approaching a maximum at ~1%,
6. at low doses, 200 ergs/mm^2, the rate of formation is about 3 times less at 77°K than that of thymine dimer at 298°K.

The first four properties are similar to those found for the spore photoproduct.

21.18 PYRIMIDINE DIMER FORMATION IN POLY T AT 77°K

R. O. Rahn J. L. Hosszu

Photoproduct formation in poly T at 77°K was investigated for the polymer dissolved in 50% ethylene glycol solutions. Absorbance measurements were made at 298°K following irradiation at 77°K. For irradiation at 280 nm, poly T in 0.05 *M* NaCl, which underwent 74% conversion to the dimer at 298°K, showed only 24% conversion at 77°K (Fig. 21.18.1). However, the initial rate of change in absorbance was 5 times faster at 77 than at 298°K. This result is consistent with a model in which (1) at 77°K only a fraction of the bases are suitably oriented to form dimers and (2) the quantum yield of dimer formation for those bases which can form dimers is an order of magnitude higher at 77 than at 298°K. By successively thawing, refreezing, and reirradiation, 70% conversion to the dimer was obtained. Presumably, more bases are made available for dimer formation by refreezing. For irradiation with 2537 A, the photosteady-state distribution at 77°K was shifted towards the dimer, since reirradiation at 298°K leads to an *increase* in absorbance.

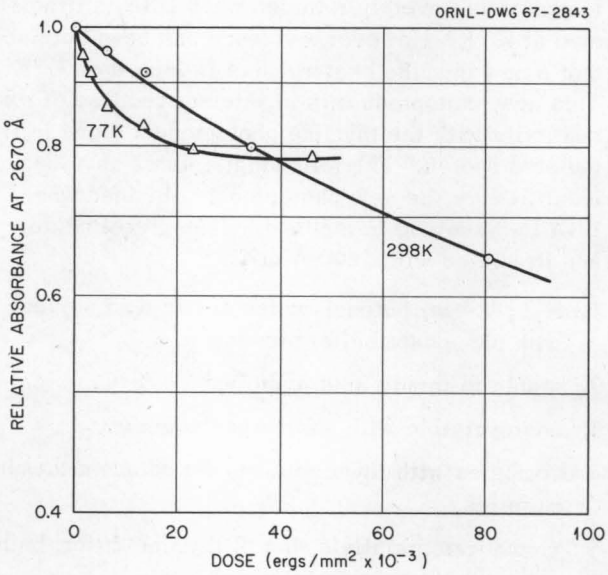

Fig. 21.18.1. Relative Change in Absorbance of Poly T in 50% Ethylene Glycol as a Function of Dose at 298 and 77°K. Irradiation at 2800 A.

21.19 ELEMENTAL COMPOSITION OF TISSUES AS A LIMITING FACTOR IN THE SPECIFICATION OF DOSE OF IONIZING RADIATION

M. L. Randolph

Introduction. – Estimation of the dose of ionizing radiation received by a biological tissue is normally obtained by multiplying a physically measured value of exposure or of dose in a radiation detector by a factor supposedly compensating for the difference in energy absorption of the detector and tissue of interest arising from differences in their compositions. The elemental composition of the tissue is usually taken as equivalent to that of muscle[1] except for the irradiation of bone or fat or irradiation by neutrons. The results of a study on the uncertainty introduced in dose estimates by the assumption that tissues are dosimetrically equivalent to muscle are presented here.

Results. – To first approximations wherein molecular binding effects, density of irradiated media, and relativistic effects are ignored, relative doses delivered to different media by various mechanisms of physical interaction can be calculated from atomic numbers (Z_i) and concentrations (p_i) of the elements (i) composing the media and their overall electron concentrations (n_0). The dose per unit exposure for the photoelectric effect was taken as proportional to

$$\sum_i \frac{p_i Z_i^4}{A_i} ;$$

for the Compton effect as proportional to n_0; for pair production,

$$\sum_i \frac{p_i Z_i^2}{A_i} ;$$

for collision losses by charged particles, n_0; for radiative losses by high-energy electrons,

$$\sum_i \frac{p_i Z_i (Z_i + 1.3)}{A_i} ;$$

and for elastic scattering of fast neutrons, 0.9 [H] + 0.1, where [H] is the concentration of hydrogen. For a number of tissue compositions and a few molecules of potential interest (culled from literature too extensive to cite here in detail) the relative doses in

Table 21.19.1. Dosimetric Significance of Elemental Composition

Absorbing Medium (Reference)	Relative Doses				
	Photoelectric Effect	Compton Effect and Collision Losses of ± Particles	Pair Production	Radiative Loss of Fast Electrons	Fast Neutrons
"Tissues"					
Muscle (ICRU, 1964)[a]	1.00	1.00	1.00	1.00	1.00
(Yamamoto, 1963)	0.91	1.00	0.99	0.99	0.98
Standard man (ICRP, 1955)	~1.35	1.00	1.06	0.99	0.98
Water	0.99	1.00	1.02	1.02	1.08
Bone (ICRU, 1964)	3.7	0.97	1.30	1.24	0.67
Fat from rat (Yamamoto, 1963)	0.6	1.01	0.85	0.88	1.11
Liver – av chicken and cat (Bioch. Handbook)	0.91	1.00	0.97	0.98	1.01
Lens, ox (Bioch. Handbook)	0.93	1.00	0.97	0.99	0.95
Egg yolk, hen (Bioch. Handbook)	0.83	1.00	0.94	0.94	1.04
Silkworm, pupae (Murakami, 1966)	~1.2	1.00	1.00	1.01	1.00
larvae, day 0 (Murakami and Kondo, 1964)		0.97	1.05	1.02	0.87
larvae, day 7 (Murakami and Kondo, 1964)	~1.2	1.00	1.02	1.02	1.03
Tradescantia inflorescences (Conger and Giles, 1950)	1.02	1.00	1.02	1.01	1.03
Vicia faba root tips (Neary, 1959)	1.07	1.00	1.00	1.01	1.02
Whole seeds – radish (Troitskii and Arramko, 1964)		0.99	0.94	0.94	0.93
wheat (Troitskii and Arramko, 1964)		0.98	1.03	1.01	0.79
"Molecular species"					
DNA (approx)	1.36	0.94	1.04	1.02	0.46
Proteins (range of several)	0.6–0.9	0.96–0.98	0.87–0.94	0.89–0.94	0.67–0.73
Lipids (range)	0.4–0.9	0.98–1.04	0.75–0.90	0.80–0.91	0.78–1.36
Chlorophyll a	0.60	0.98	0.84	0.89	0.81
Carbohydrates	0.78	0.97	0.94	0.95	0.69

[a]Standard tissue.

a sufficiently large homogeneous mass of the media have been calculated and are presented in Table 21.19.1.

Discussion and Conclusion. – Evidently, from the ranges of values given in Table 21.19.1, only for the Compton effect and collision losses by charged particles is the assumption of tissue equivalence to muscle generally valid to ±2%. For collision losses in charged particle irradiations the dependence on mean ionization potential, which is not included here, may introduce further uncertainty of a similar magnitude. LET values, as usually calculated in Mev cm^2 g^{-1} for water, have the same dependence on composition and ionization potential as do collision losses of charged particles. Values given for molecular species are only suggestive rather than directly applicable. The overall conclusion is that unless detailed consideration is given to elemental composition of the tissue volume of interest, which is the tissue of interest and a surrounding layer of thickness approaching the range of ionizing secondaries produced, the uncertainties of composition make meaningless to radiobiology physical measurement or dose specification to much better accuracy than ±2% even under favorable irradiation conditions.

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Cooperative Programs

AEC-NIH CHEMICAL CARCINOGENESIS

Biochemistry of Carcinogenesis (22.1)

Stanfield Rogers
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Enzymology of Chemical Carcinogenesis (22.2–22.7)

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E. L. Murray[b]

Mammalian Chemical Carcinogenesis (22.8, 22.9)

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Chromosomal Effects of Chemicals and Radiation (22.10)

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Effects of Radiation and Chemicals on Paramecium (22.11)

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Biophysics of Carcinogenesis (22.12–22.14)

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Chemical Mutagenesis in Microorganisms (22.15)

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Inhalation Carcinogenesis (22.16–22.22)

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Biochemical Regulation (22.23–22.25)

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22.1 STUDIES WITH CERTAIN ONCOGENIC AND PLANT VIRUSES

Stanfield Rogers

Induction of a Lipoprotein by Mutant Shope Papilloma Virus Recoverable in Domestic Rabbits. – In efforts to purify this virus for comparison with the wild type, a bandable protein in a 25 to 50% sucrose gradient not occurring in domestic-rabbit papillomas induced with wild-type virus was encountered. Sporadic low infectivity was found associated with the band. Its amino acid composition is considerably different from the protein coat of wild-type virus. The density of the band is much less than the density of banded wild-type virus. The percentage nitrogen in the protein is only 8% as compared with about 16% for the wild-type virus protein coat. The pellet stains with Sudan IV, indicating the presence of lipid. Electron photomicrographs made by Dr. Harris of the Molecular Anatomy Group revealed structures containing no capsomeres and resembling most tiny fat droplets. Thinking that this lipoprotein might be nonspecifically absorbed on the surface of infective virus DNA, and thereby protecting it to a degree to make it infective using standard inoculation procedures, the DNA obtained from purified wild-type virus was incubated with this lipoprotein. It had no effect upon the infectivity of the DNA. It is not yet clear whether the infectivity of extracts of these domestic-rabbit recoverable line papillomas is the result of infective DNA somehow protected from DNAase or whether this lipoprotein is forming the protein coat of the virus.

Does the Mutation Responsible for Virus Recoverability in Recoverable Line Papillomas Involve the Structure of the Arginase Induced by the Virus?[1] – In metabolic studies of the papillomas induced by the mutant virus, the arginase activity of the tumors is about half that found in tumors induced by wild-type virus. The free arginine pool in these tumors is considerably higher than the pool in tumors induced by wild-type virus. That the growth rate of the recoverable line tumors is much less than those induced with wild-type virus adds further weight to our conclusion that the low arginine pool in Shope-virus-infected cells is the cause of the depletion of arginine-rich histones in the papillomas, which in turn derepresses DNA and allows thereby for the rapid growth of the cells.[2,3] The arginine pool in normal squamous cells of the rabbit is much higher than in the tumors induced by either of these two virus lines. The lysine-arginine ratio in normal skin is 1 : 40, in wild-type papillomas 15 : 1, and in recoverable line papillomas 4 : 1.

That the arginase activity of the papillomas induced with the recoverable line virus was less raised the question as to whether the number of enzyme molecules is less or whether the enzyme had been rendered less effective because of the mutation. Should this latter be the case, it would prove unequivocally that the information of the synthesis of the enzyme is virus information. Though our chemical and immunological data strongly indicate that this is so,[4,5] it remains inferential to a degree. Efforts were therefore made to purify the arginase induced with the recoverable line virus and to compare it with the enzyme induced with wild-type virus. The purification of the recoverable line virus induced enzyme is not complete, at least in the quantities required for peptide analysis. However, we already have found that it moves differently on carboxymethyl cellulose than does the enzyme induced with wild-type virus. Whether the structural difference responsible for this difference in elution from the column will be reflected in a change in the peptide pattern and amino-acid content will be seen shortly.

Efforts to Put Synthetic Information on the Tobacco Mosaic Virus RNA. – The RNA of this virus was kindly supplied by Dr. C. A. Knight of the Virus Laboratory at Berkeley. Both ^{14}C and cold UDP have been used in conjunction with polynucleotide phosphorylase obtained from commercial sources and derived from Micrococcus lysodeikticus. The enzyme from this source requires an RNA primer. In the first tests 8000 counts min^{-1} cm^{-3} were found associated with the TMV-RNA. Biological tests in Xanthi tobacco plants revealed the RNA remained infective. Tests under way on a larger scale and using both Xanthi and Turkish tobacco plants are being carried out to find whether there is detectable polyphenylalanine in the infected leaves and whether in the systemic infection of the Turkish variety, the new systemically transmitted TMV will also have polyphenylalanine in the leaves not inoculated. If so, it would be indicative that the RNA with polyuridine added was being copied with polyuridine still incorporated. The hunt for polyphenylalanine is being done in conjunction with Dr. Pfuderer of the Enzymology Group.

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22.2 ETHIONINE CARCINOGENESIS

Beryl Ortwerth G. David Novelli

When ethionine, an analogue of methionine, is added to the diet of rats for five months, hepatomas will develop in almost all of the animals.[1] It has also been shown that the nucleic acids of rat liver, especially the sRNA fraction, will become labeled shortly after the injection of ^{14}C-ethyl-labeled ethionine.[2] In an attempt to correlate these observations, a kinetic and chromatographic comparison was made between crude tRNA fractions isolated from normal liver and hepatoma tissue.

Results. – Preparations of tRNA isolated from rat livers following a 48-hr exposure to ^{14}C-ethyl-labeled ethionine were subjected to both reverse-phase and Freon chromatography.[3,4] These preliminary separations indicated that there are certain tRNA species which are ethylated to a greater extent than others. This is in contrast to the random labeling patterns obtained with ^{14}C-methyl-labeled methionine under the same conditions.

Crude tRNA fractions have also been prepared from the livers of normal rats and rats fed the ethionine-containing diet for five months. Preliminary measurements of aminoacyl-accepting capacity for ten different amino acids have been carried out on the above tRNA preparations. No difference in amino acid accepting ability was observed with five of the amino acids tested; however, with the others the tRNA from the ethionine-fed rats showed a 10 to 30% decrease in total charging. This also suggests that the effects of ethionine on tRNA are variable. Currently the changes in the individual tRNA species due to the ethionine feeding are being investigated. This is accomplished by cochromatographing the two tRNA preparations which have been precharged with ^{14}C- and ^{3}H-labeled amino acids.

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22.3 STUDIES ON AMINO ACID ACTIVATION IN MOUSE PLASMA CELL TUMORS

Wen-Kuang Yang G. David Novelli
C. J. Wust[1]

Introduction. – The origin of immunological specificity and physicochemical heterogeneity of immunoglobulins has been the subject of various hypotheses. Recently a possible control mechanism of immunoglobulin biosynthesis was discussed,[2,3] although no experimental proof or disproof is available. In the present investigation, cell-free amino acid activation is being studied as the first step of a systematic research on this problem, because the multiplicity and individual specificity of various amino acid tRNA-synthetase systems possibly provides a candidate for the control mechanism. As the plasma cell tumor produces a rather homogeneous population of myeloma protein which is similar to the immunoglobulins and for which the primary structure has been extensively studied, this kind of functional tumor was chosen for study.

Results and Discussions. – Mouse mineral-oil-induced plasma cell tumors, MOPC-31B and MOPC-31C, supplied by Dr. M. Potter of the NIH, have been successfully maintained by subcutaneous transplantation in Balb/cum mice. The genetic constancy of these tumors in myeloma protein production has been ascertained by immunoelectrophoretic analysis using specific antisera prepared against L-chain and papain Fc fragment of the myeloma protein. Ascites forms of these tumors have also been produced.

Transfer RNA was prepared by phenol extraction, 1 *M* NaCl extraction, and DEAE-cellulose chromatography. Initial homogenization of the tissue in hypertonic sucrose medium and removal of nuclei were found necessary for the prevention

of DNA contamination. Synthetase preparations of the liver and tumor tissues were made by conventional methods except that 10% glycerol was added to the homogenizing medium and that Sephadex G-100 gel filtration was performed. The synthetase preparations were found to be very active for all 19 amino acids tested. The optimal Mg^{2+}/ATP ratio of the synthetase was determined for every amino acid and found to be different from other systems, such as those of *E. coli* and yeast; especially remarkable for the tumor synthetases are those of leucine (0.5 to 1) and glutamate (2.0 to 2.5). When the amount of incorporated 19 ^{14}C-amino acids and added tRNA were calculated on a molar basis, a 50 to 55% value was obtained. Chromatographic studies of tumor tRNA on Freon columns[4] have been initiated. Good separation was obtained both at pH 4.5 and 7.5. A simple direct assay on the filter paper disks was devised for screening chromatographic fractions for the amino acid accepting ability of tRNA. Preliminary results showed that from the pH 4.5 Freon column four to five leucine tRNA's, three lysine tRNA's, and two histidine tRNA's were observed. Cochromatography of precharged ^{14}C- and ^{3}H-aminoacyl tRNA will be performed on the column with samples from MOPC-31B and 31C tumors to see if any change in tRNA can be related to the production of mouse Bence-Jones protein.

As a sideline of interest, mouse plasma cell tumor tissue was found to have a soluble system of arginine incorporation into protein, which is much more remarkable than the system found in the rat liver.[5] Further studies will be performed aiming at an understanding of the physiological meaning of this phenomenon.

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22.4 PHYSIOLOGICAL PARAMETERS OF L-M CELLS PROPAGATED IN VIVO AND IN VITRO

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G. David Novelli

The capacity of the L-M cell to initiate tumor formation in vivo when inoculated into C3H (Andervont substrain) mice has provided a means of obtaining large quantities of tissue for biochemical studies. Studies of uridine and thymidine incorporation, alkaline phosphatase activity, chromosomal characteristics, and growth rate of the cell population have been undertaken. The results suggest that, although one loses a small portion of the L-M marker chromosomes, other morphological and biochemical characteristics remain relatively stable. The only biochemical instability encountered is apparently sex dependent. The increased uridine incorporation observed in L-M cells obtained from tumor-bearing female mice is correlated with a decrease in the percentage of tumor regression.

In addition to the studies of tumorigenesis, the large-scale cultivation of L-M cells in vitro has continued with the result that consistent harvests of approximately 2 g/liter are obtained. The routine propagation of cells in 5-liter volumes contained in a modified New Brunswick fermentor has been established. As a result, systems are now available for obtaining material in sufficient quantities for comparative studies of the in vitro and in vivo state of the L-M cell.

References

[1]Mammalian Genetics section.

[2]Pathology and Physiology section.

22.5 LEUCYL-tRNA FROM T2-INFECTED *E. COLI* B

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G. David Novelli

It has been reported[2] that leucine tRNA from *E. coli* which had been infected for 2 hr with T2 phage is chromatographically distinct from that isolated from normal or 6-min-infected cells. The main distinction is that two new peaks, IV and V, appear in the 2-hr infection. It was previously

reported[2] that these peaks (IV and V) are present in normal cells but at a very low level. Further evidence showed that only three peaks, I, II, and III, were observed with leucyl-tRNA from normal *E. coli* under the chromatographic conditions used. Therefore current studies have been efforts to elucidate problems concerning the origin and function of peaks IV and V of leucyl-sRNA, which appear to be unique to T2-infected *E. coli*.

Timing studies show that the new peaks appear as early as 25 min after infection. The addition of chloramphenicol (to inhibit protein synthesis) and fluorodeoxyuridine (to inhibit DNA synthesis) 6 min after infection does not prevent the appearance of the new peaks. Chloramphenicol added 3 min prior to infection does prevent their appearance. These results suggest that protein synthesis early in the infectious cycle is necessary for the appearance of the new peaks. They also rule out the possibility that the new peaks are an artifact of isolation in the presence of large amounts of DNA. Studies with methyl-deficient tRNA rule out undermethylation as the cause of the appearance of the new peaks.

Experiments were done using ^{32}P to label the tRNA in control and infected cells in order to investigate the possibility of de novo synthesis. The results were ambiguous, possibly due to contamination with rapidly labeled mRNA and turnover of the $-C-C-A$ terminal groups in the tRNA.

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22.6 STUDIES ON THE PURIFICATION AND CHARACTERISTICS OF tRNA PYROPHOSPHORYLASE

Audrey N. Best G. David Novelli

Introduction. – We have continued our investigation into the properties of the enzyme(s) that incorporates AMP and CMP from ATP and CTP into the terminal position of tRNA:

$$\text{tRNA----} + \text{ATP} + 2\ \text{CTP} \rightleftharpoons \text{tRNA ----pCpCpA} + 3\ \text{PP}.^{1,2}$$

The substrate tRNA for the initial assays of the enzyme has been prepared from a partially purified tRNA fraction from *E. coli* w by periodate oxidation followed by amine cleavage and subsequent treatment with alkaline phosphatase. When the enzyme has been purified to the desired degree of homogeneity, it will be studied extensively and characterized, and the pure preparation can be of use in the investigations of various tRNA's.

Results and Discussion. – An enzyme preparation from *E. coli* B that incorporates AMP (from ATP) into oxidized tRNA can be purified approximately 100-fold by chromatography on diethylaminoethyl cellulose (DEAE) and hydroxylapatite, but the preparation was not of the desired purity. Preliminary experiments have indicated that chromatography on DEAE followed by chromatography on carboxymethyl cellulose might result in a more purified preparation. Experiments are now in progress to improve the present chromatographic procedures and to expand them for large-scale production of the enzyme.

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22.7 ISOLATION OF THE PRODUCT FORMED BY THE SOLUBLE AMINO ACID INCORPORATING SYSTEM

Lawrence Rosen Shigemi Iiyama
G. David Novelli

Introduction. – The soluble system which incorporates phenylalanine into a hot TCA-insoluble product is resistant to RNAase and is found in the high-speed supernatant from trout liver homogenate.[1] The product which is formed is totally excluded by Sephadex G-50, and more than 80% of the incorporated radioactivity can be recovered by passage through G-50.[2] This report deals with the isolation of the radioactive product or products.

Results and Discussion. – Overnight storage at 2° of the excluded G-50 fraction (peak A), which contains the incorporated phenylalanine, leads to a solubilization of 50 to 70% of the TCA-insoluble

product. This is not unexpected, since passage through G-50 removes phenylalanine and ATP and the removal of only the latter leads to a rapid degradation of the product.[2] This is the reason that the incorporated radioactivity is not completely recovered by passage through G-50, since removal of free phenylalanine and ATP is accomplished before the G-50 fractions can be assayed for TCA-insoluble radioactivity. In spite of this difficulty the following procedure was used to separate two stable radioactive fractions from peak A.

After overnight storage at 2°, peak A is again passed through Sephadex G-50. Two radioactive peaks, accounting for more than 90% of the radioactivity, are recovered: an excluded fraction appearing just after the void volume, peak A1, and a fraction, peak A2, which appears near one bed volume. More than 80% of the radioactivity in peak A1 is TCA-insoluble, whereas peak A2 is entirely TCA-soluble. Fractionation of peak A2 on a Biogel P2 column yields two radioactive peaks, with R_F values of 0.70 and 0.48. In two separate experiments, the radioactivity in the peak eluting earlier ($R_F = 0.70$) accounted for more than 90% of the recovered radioactivity. An R_F of 0.7 on Biogel P2 indicated a molecular weight greater than 600, based on an R_F of 0.48 for phenylalanine, which is the same R_F as exhibited by the second peak, which also moves identically on paper with ^{12}C-phenylalanine using *n*-butanol: acetic acid: water[3] as the developing solvent.

Exclusion by Sephadex G-50 indicates that the molecular weight of peak A is greater than 10,000, which suggests that part of the protein fraction necessary for activity serves as an acceptor molecule for phenylalanine, as has been found for the soluble system purified from *E. coli*, which utilizes an acceptor protein molecule.[4] Suggestive evidence for this has been the isolation of a crude ammonium sulfate fraction, inactive by itself, which increases the extent of incorporation when added to an active preparation.

With respect to the other radioactive product ($R_F = 0.70$ on Biogel P2) the data demonstrate that it represents a breakdown product from originally TCA-insoluble product. It may be composed of heterogeneous molecules all of approximately the same molecular weight. Concentration of this material under reduced pressure at room temperature followed by chromatography in *n*-butanol: acetic acid: water shows the presence of free phenylalanine and two other radioactive peaks. This indicates that some of the radioactivity in peak A2 is a bound form of phenylalanine which is labile to the conditions of chromatography.

A plausible interpretation of the data is that peak A breaks down to peak A1 and a smaller fragment (peak A2), but other possibilities cannot be excluded. Such a possibility is that peak A1 is a stable component of peak A and did not arise from the degradation of large radioactive molecules in peak A, although peak A2 does arise from such degradation. These results indicate that the incorporation and degradation reactions of the present system are more complex than a simple incorporation of phenylalanine followed by its removal from product as free phenylalanine.

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22.8 PRELIMINARY OBSERVATIONS ON THE COMBINED LATE SOMATIC EFFECTS OF X RAYS AND TRIETHYLENE MELAMINE IN MICE

J. W. Conklin W. D. Gude[1]

Introduction. – The radiomimetic alkylating agent triethylene melamine (TEM) has been found to resemble x rays in certain late somatic effects on RF mice.[2] These include shortening of the life-span, leukemogenic effects on the thymus, the induction of tumors in the lung, ovary, and other organs, and the formation of lens opacities. The present study was undertaken to explore the combined effects of TEM and x rays administered concurrently, with the aim of determining whether the effects of the two agents would be additive to a degree consistent with a common mode of action.

Results and Discussion. – Ten-week-old female RF/Un mice were subjected to: (1) I-P injection of TEM, 0.25 to 4.0 mg/K; (2) 25 to 500 r whole-

body 300-kvp x irradiation (80 r/min); or (3) both TEM and x irradiation (TEM injected 1 to 5 min before irradiation). Preliminary results 20 months after treatment show that each agent given alone shortened the life-span, the mean survival time decreasing from about 20 months in the controls to about 13 months in mice exposed to 500 r of x rays, and to about 16.5 months in mice injected with 4.0 mg/K of TEM. In mice receiving both agents, the extent of life shortening exceeded that induced by a comparable total dose of either agent alone, especially at high dose levels (Fig. 22.8.1). Likewise, each agent alone induced thymic lymphomas, the incidence rising from 4% in controls to 24% in mice exposed to 400 r and to 14% in mice injected with 4.0 mg/K TEM; when both agents were administered together, the incidence exceeded that induced by either agent alone, and the dose-incidence curve showed less tendency to plateau at high dose levels than it did with either agent alone (Fig. 22.8.2).

These results indicate that the combined life-shortening and leukemogenic effects of the two agents administered together were additive. The results suggest, moreover, that the combined leukemogenic effects of the two agents were more additive than the combined cytotoxic effects, causing the agents to be synergistic in their leukemogenic action on the thymus at high dose levels. Since, however, other types of lesions induced by the two agents remain to be analyzed, the significance of the combined effects in terms of possible mechanisms is yet to be determined.

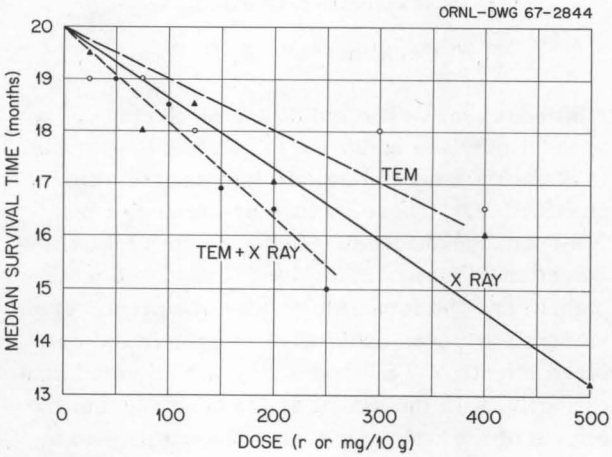

Fig. 22.8.1. Influence of X Rays and TEM Administered Separately or Together on the Longevity of RF Mice.

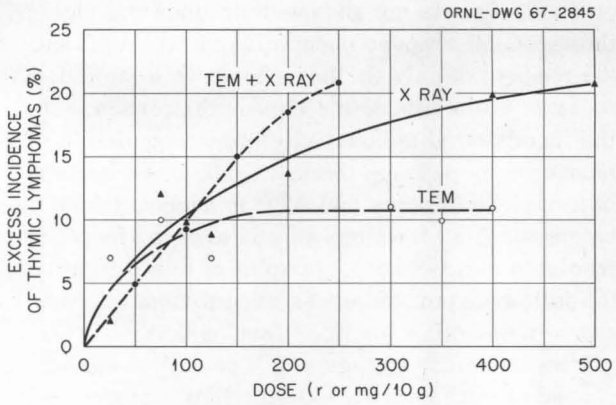

Fig. 22.8.2. Influence of X Rays and TEM Administered Separately or Together on the Incidence of Thymic Lymphoma in RF Mice.

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22.9 LEUKEMOGENIC EFFECTS OF METHYLCHOLANTHRENE ON THE MOUSE THYMUS

Lou C. Satterfield[1] Mildred G. Hayes[1]
A. W. Craig[2]

Introduction. – The combined administration of methylcholanthrene and croton oil has been reported by Irino *et al.*[3] to cause a high incidence (50%) of myeloid leukemia in RF mice, having an average latency of two to three months. This observation contrasts with our failure to induce myeloid leukemia in RF mice with radiomimetic chemicals, although we have been able to induce it readily with x rays. To investigate the action of methylcholanthrene in RF mice of the Oak Ridge subline, we have attempted to repeat the experiments of Irino *et al.*

Results and Discussion. – Approximately 50 RF mice of each sex were treated as follows: 0.1 ml of an 0.8% solution of 20-methylcholanthrene in olive oil was given orally via stomach tube three times a week. Twice a week a shaved area on the back of each animal was painted with a solution of 5% croton oil in benzene, using a No. 8 brush. The

treatment was begun when the animals were six weeks old and continued until death. Fifty-three percent of the mice died with thymic lymphomas at 14 to 34 weeks of age and 7% with nonthymic lymphomas or reticulum-cell sarcomas at 14 to 28 weeks of age (Fig. 22.9.1). Forty percent of the animals died without leukemia at 8 to 34 weeks of age, primarily from abscesses associated with the trauma of treatment. The leukemia mortality in untreated control RF mice was less than 1% and the total mortality about 4% for a comparable time period. Almost all the animals developed papillomas up to 5 mm in diameter in the area of croton oil treatment. The papillomas ranged in numbers from one to two in mice dying with a short latency to 30 or more in mice dying after several weeks of treatment.

Although these results fail to confirm the induction of myeloid leukemia in RF mice by treatment with methylcholanthrene, they do reveal the induction of thymic lymphomas in large numbers. The difference between the types of leukemia induced in our RF mice and those predominating in mice of the Japanese RF subline remains to be explained.

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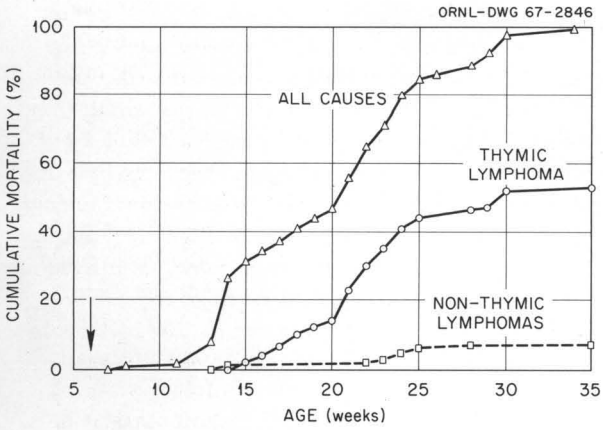

Fig. 22.9.1. Cumulative Mortality from Leukemia and Other Causes in Mice Treated with Methylcholanthrene and Croton Oil. Arrow denotes age at start of treatment.

22.10 SELECTION FOR GENETIC VARIANTS IN MAMMALIAN SOMATIC CELLS IN VITRO

E. H. Y. Chu E. Virginia Merriam
Patricia A. Brimer

Various selective techniques have been employed in an attempt to isolate variants from an established line of Chinese hamster cells in culture. The parental cells have a near-diploid constitution (23 chromosomes), an average generation time of about 15 hr, and 70% plating efficiency.

Taking advantage of the fact[1] that hydroxyurea (HU) at appropriate concentration kills these cells during DNA synthesis but not at other stages of the cell cycle, clones with altered nutritional requirements might be obtained by exposing cells alternately to normal growth medium and to that containing HU but deprived of one exogenous metabolite. We observed in two experiments that one round of HU treatment killed 50% of the population and two rounds further reduced the surviving fraction to 17%. The elimination of wild-type cells can be made more effective by increasing the number of such cyclic treatments and by mitotic synchronization prior to the HU exposure.

The second selective technique used was the "thymineless death" method of DeMars and Hooper.[2] During the period of thymine deficiency created by aminopterin (AP), prototrophs would die, whereas auxotrophs cease to grow and thus are spared from killing in the absence of a particular metabolite. The auxotrophs resume growth when returned to normal medium without AP.

To date, selections have been made for proline (a nonessential amino acid for mammalian cells) autotrophs as well as for variants that presumably fail to effect the enzymatic conversion of citrulline to arginine (an essential requirement). Experiments are in progress to test and characterize, both genetically and cytologically, these clonal isolates.

Work has also begun in the third type of screening involving the recovery of drug-resistant variants. It is hoped that these variants, if proven to be mutants, would serve as useful markers for cytogenetic studies such as mutagenesis and somatic cell hybridization.

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22.11 COMPARISON OF MUTATION INDUCTION BY X RAYS, ULTRAVIOLET RADIATION, TRIETHYLENE MELAMINE, AND NITROSOGUANIDINE

R. F. Kimball
Barbara R. Bussell
E. G. Bailiff
Stella W. Perdue[1]

Introduction. – Previous work[2] with x rays and with the trifunctional alkylating agent triethylene melamine (TEM) has led to the conclusion that at least part of the mutations induced by these agents arise from premutational damage that is not fixed as final mutation until DNA synthesis and is subject to elimination, probably by repair, before fixation. Evidence for the existence of this mechanism is provided by postmutagen treatment with streptomycin. This treatment reduces the mutation yield if started before DNA synthesis but is ineffective after DNA synthesis. Less direct evidence is provided by the observation that the longer the interval between exposure to the mutagen and DNA synthesis, the less the mutation yield. Presumably the rate of decrease of mutation as the time to DNA synthesis increases is a measure of the rate of elimination of premutational damage. Both kinds of evidence have now been obtained for two other mutagens – 2537-A ultraviolet radiation (uv) and the chemical mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NG).

Results and Discussion. – The mutation yields for uv and NG are decreased by posttreatment with streptomycin just as are those with x rays and TEM. The curves relating mutation yield to time of treatment in the cell cycle are shown in Fig. 22.11.1 for uv and NG, with lines representing the previously published curves for x rays and TEM for comparison. Cells treated just before DNA synthesis (2 to 3 hr after division) show the maximum yield, presumably because there is a minimum time for elimination of premutational damage. Cells treated just after DNA synthesis show a minimum yield, in part at least because the time for elimination of damage is long. The uv and NG curves are very similar and suggest that repair is rapid enough so that nearly all damage is eliminated in cells treated just after division. Repair seems to be slower with TEM, so that appreciable mutation yields are obtained by treatment in the latter part of the division cycle. The x-ray data are more complex, showing among other things a peak yield in those cells treated during midmitosis. This suggests that x rays, unlike the other three mutagens,

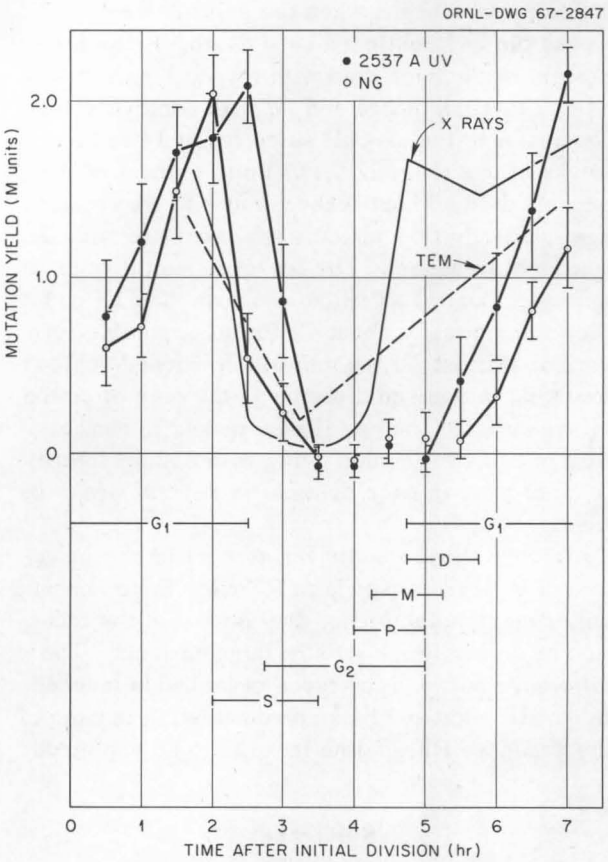

Fig. 22.11.1. Mutation Yield with Time of Treatment in the Cell Cycle.

induce some mutation by processes that have other end points than DNA synthesis. It may also be that repair of x-ray-induced damage occurs at different rates in different stages of the cell cycle.

The four mutagens must differ from each other completely in their initial interaction with DNA and possibly in the kinds of mutations which they produce. Yet they all have in common that a considerable part of all the mutation they produce results from initially reparable premutational damage that is fixed as final mutation at DNA synthesis. Despite this similarity there are obvious differences in details, such as apparent rates of repair, that make the sensitivity pattern during the cell cycle different for these four mutagens.

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22.12 TALES OF TYROSINE IN POLYMERS

J. W. Longworth

Is There a Second Tyrosine Component in the Luminescence of Bovine Serum Albumin? – Tryptophan absorbs and fluoresces to the red side of tyrosine; hence its contribution to an emission spectrum can always be measured and removed from an emission where both tyrosine and tryptophan contribute, leaving only the tyrosine component. The tyrosine part of the fluorescence of B.S.A. at pH 7 and 298°K consists of two bands, one at 304 nm and a shoulder to longer wavelengths. In acid-denatured B.S.A. there is only the 304-nm band. If the acid tyrosine profile is normalized at 295 nm to the neutral tyrosine bands and subtracted, then a band is found at 340 nm. This is the location of the tyrosinate anion fluorescence.

The excitation spectra of the 305- and 340-nm tyrosine contribution are essentially similar to neutral tyrosine absorption. Though the peak of the 350-nm band lies to the red of the 305-nm band, the latter appears to extend to the same long-wavelength edge. The interpretation is that the 350-nm peak originates in a "buried" tyrosine, which looses the hydroxyl proton in the excited state to the carboxylate cation paired with it. The 305-nm fluorescence is from both "external" and "buried" tyrosines. Similar 350-nm peaks are seen from the "photoreactivating enzyme" and amino-acyl synthetase, though not from ribonuclease (where the "buried" tyrosines are believed to be nonfluorescent).

The Cause of the Red Shift in the Phosphorescence of Tyrosine Residues in Ribonuclease. – RNase at pH 7 has a small fluorescence quantum yield compared with tyrosine at both 298 and 77°K. Its phosphorescence is red-shifted to 410 nm from that of tyrosine at 390 nm. Identical behavior is found for poly-L-tyrosine and for oxytocin, which has only one tyrosine. The model compound L-tyrosyl-diketopiperazine, which has a small quantum yield at 298°K, behaves identically to tyrosine at 77°K. Since the luminescence of RNase most probably originates in the "external" tyrosines, and the x-ray structure indicates that they do not lie near each other, it is hard to offer an explanation for the differences found between tyrosine and RNase, as is also the case in the monopeptide oxytocin, other than the common presence of tyrosine next to an S-S bridge.

Enzyme-Inhibitor Complex of Ribonuclease. – ATP is a competitive inhibitor for RNase, and forms a complex which from uv difference spectra resembles that of protonated N_1 ATP. The neutral form of ATP luminesces, whereas the protonated ATP at 77°K is nonluminescent, but at 298°K fluoresces at 390 nm. Provisional observations on the complex at pH 5.2 are (*a*) fluorescence of tyrosine increased at 298 and 77°K; (*b*) phosphorescence of tyrosine quenched tenfold; (*c*) no 390-nm fluorescence at 298°K; (*d*) 30% quenching of ATP phosphorescence and 2 nm red shift; (*e*) no ATP fluorescence at 77°K. Hence there is most certainly an interaction between ATP and RNase tyrosines – furthermore, tyrosine 115 is at the active site, but remote from the other "external" tyrosines 73 and 76. One could suggest that there is a complex formed between ATP and tyrosine 115, with energy transfer from tyrosines 73 and 76 to such a complex, but clearly a more detailed investigation is essential before any interpretation can be confidently made.

22.13 X-RAY CRYSTALLOGRAPHY

J. R. Einstein C. H. Wei

Instrumentation. – 1. Instrumentation for the automation of x-ray diffraction data collection has been installed. The system utilizes a PDP-8 computer (Digital Equipment Corporation) as the controlling element. Programs for the various diffractometer functions are stored in the computer memory. Commands to initiate the various programs, and necessary parameters, are transmitted by the operator to the computer via a teletype unit, which is also used for data output. The system was designed principally by Dr. W. R. Busing of the Chemistry Division of ORNL and was assembled and installed by the Instrumentation and Controls Division, with Mr. R. T. Roseberry as supervisor.

2. Work is in progress on the design and fabrication of a cooling system for maintaining protein crystal specimens at finely controlled low temperatures, while mounted on the diffractometer. Assistance has been given by members of Bio-Engineering, the Machine Shop, and the Instrument Engineering Department of Y-12.

Structure Studies. – 1. Two photoproducts, thymine dimer and 1-methyl thymine dimer, were furnished by J. W. Longworth, R. O. Rahn, and J. L. Hosszu. Although our attempts to prepare HCl and

HBr addition products for these compounds have not been successful, crystal data for 1-methyl thymine dimer were obtained, as follows. Space group $P2_1/c$, $a = 7.50$ A, $b = 6.20$ A, $c = 14.43$ A, $\beta = 117°$, $Z = 2$. Intensity data are to be collected on the automated diffractometer, and the structure is to be determined either by direct methods or by the application of Patterson and Fourier methods.

2. Crystals of BUpT photoproduct, made available to us by Dr. R. J. Drewer of the California Institute of Technology, were examined with Weissenberg and precession camera photographs. The crystals, which are highly mosaic, were shown to have orthorhombic symmetry, with probable space groups $I222$, $I2_12_12_1$, $Imm2$, and $Immm$. In an attempt to obtain a suitable single crystal for further investigation, the sample was recrystallized from ethanol solution. It is planned that an intensive examination be carried out on this material.

3. Molecular and crystal structures of a series of bis(*N-t*-butylpyrrole-2-aldiminato) M(II) (where M = Co, Ni, Cu, and Zn) complexes[1] have been previously determined[2] systematically from data collected by the multiple-film technique. An isotropic least-squares refinement of the nickel complex, however, resulted in unusually high thermal motions for two of the butyl carbon atoms. In order to obtain data of higher accuracy, a complete three-dimensional set of counter data was collected using the automated diffractometer in the Chemistry Division of ORNL (courtesy of Dr. H. A. Levy). Processing of the data is in progress. A detailed comparison will be made of structural features based on the two different sets of data.

The refinement of the structure for bis(*N-t*-butylpyrrole-2-aldiminato) Cu(II) (tegragonal) was completed by one of us (CHW).

4. Three-dimensional film data for a tetrameric cobalt-carbonyl mercapto complex $[Co(CO)(SC_2H_5)_2]_4$ were previously collected at the University of Wisconsin. Structural analysis was carried out at the Biology Division.[3]

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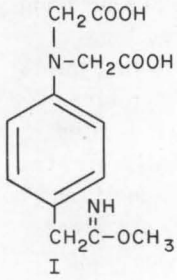
22.14 INCORPORATION OF HEAVY ATOMS INTO PROTEINS

F. C. Hartman

The present work is directed toward the development of chemical methods for incorporating heavy atoms into proteins at specific sites to facilitate the elucidation by x-ray diffraction of three-dimensional protein structure.

Approach. – (*a*) To synthesize chelating reagents containing functional groups capable of reacting covalently with proteins. (*b*) To synthesize protein reagents which contain covalently bonded heavy atoms.

Current Status of Work. – (*a*) Two protein reagents, which possess the features described in *a* and *b* above, respectively, have been prepared. These compounds, both of which should react specifically with protein amino groups, are methyl α-(*N,N*-dicarboxymethylanilino)-acetimidate (I) and methyl α-(*p*-iodophenyl)-acetimidate (II).



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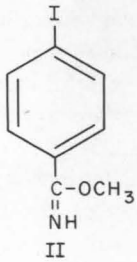

II

(*b*) Ferredoxin from *C. pasteurianum* and ribonuclease T_1 are being isolated for use as model proteins for modification with the above two reagents. These proteins have been chosen primarily on the basis of their low amino content.

22.15 THE RELATIONSHIP BETWEEN CARCINOGENICITY AND MUTAGENICITY OF ETHYLATING COMPOUNDS

H. V. Malling F. J. de Serres

The biological effect of many carcinogens depends on metabolic activation, which then leads to formation of the proximate carcinogens or ultimate carcinogens.[1] Diethylnitrosamine (DEN), which is a potent carcinogen in many rodents,

presumably needs to be metabolized before becoming tumorogenic. Metabolism results in formation of ethylating agents, and it is presumably through the formation of these compounds that DEN is able to alkylate the nucleic acids in liver cells and other organs.

The nitrosamines show mutagenic activity in *Drosophila* but not in microorganisms. All suggestions about the active metabolic product involve an initial enzymatic hydroxylative *N*-dealkylation of the nitrosamine. The lack of mutagenicity in both bacteria and *Neurospora* may be because these organisms are not able to carry out this hydroxylation. The hydroxylating process, however, can be carried out in an enzyme-free system.[2] Under these conditions, it was shown that hydroxylation of DEN led to formation of compounds which were able to induce reversions of an *ad-3B* mutant of *Neurospora crassa*.[3]

This assay, however, gives no information about the ability of these compounds to break chromosomes. In view of the potent and specific carcinogenesis of DEN, we felt that this information was important, and a separate study of the ability of DEN to induce chromosome breakage and produce mutation by chromosome deletions in Neurospora was carried out in the two-component heterokaryon system of de Serres.[4]

Since the breakdown of DEN dissolved in the enzyme-free hydroxylating system only occurs when oxygen is present, the effect of DEN and the hydroxylating mixture alone can be studied by bubbling through with nitrogen instead of oxygen.

Conidia from the heterokaryon were treated with the hydroxylating mixture with or without DEN and under either oxygen or nitrogen bubbling. After the treatment the conidia were assayed for survival and mutation induction in the *ad-3* region by the direct methods. *Ad-3* mutants were only obtained from the combined treatment of DEN dissolved in the hydroxylating mixture under oxygen bubbling. The mutation frequency after treatment with DEN + O_2 was 15.3×10^{-6} purple mutant per survivor and after DEN + N_2, 0.13×10^{-6} purple mutant per survivor. The latter was not significantly higher than the control. The survival frequencies were respectively 89 and 86%. These results confirm our first finding that hydroxylation is a necessary step in the mutagenic action of DEN.

Ethyl methanesulfonate is a potent mutagen, is able to ethylate RNA and DNA, but is a weak carcinogen.[5] If a part of the mechanisms for induction of cancer is similar to those in the inductions of mutations, the comparison of the properties of mutants induced by DEN + O_2 with those induced by EMS might provide indications for which genetic alterations are likely to lead to cancer. Unfortunately, there is as yet no analysis of EMS mutants induced in the heterokaryon. The EMS-induced mutants are obtained after treatment of the haploid wild-type strain 74-OR23-1A; with this strain *ad-3* mutation resulting from chromosome deletion cannot be recovered (Table 22.15.1).

The results show clearly that DEN + O_2 does not induce multilocus chromosome deletions. The ratio of *ad-3A* to *ad-3B* mutants is the same after treatment with DEN + O_2 or EMS. The percent of nonpolarized mutants is higher among the mutants induced by DEN + O_2 than among mutants induced by EMS and vice versa for the percent of noncomplementing mutants. The percent of leakiness is higher after treatment with DEN + O_2 than after treatment with EMS. The data indicate that a

Table 22.15.1. Distribution of *ad-3* Mutants Induced After Treatment of a Two-Component Heterokaryon of *Neurospora crassa* with DEN + O_2 or a Haploid Wild-Type Strain with EMS with Respect to Genotype, Complementation Group, and Leakiness

	Frequency (%)	
	DEN + O_2	EMS
Chromosome deletions (AB; ABnic, Bnic)	0	*a*
Point mutations		
ad-3A	28.2	24.6
ad-3B		
NP (nonpolarized)	45.7	27.8
P (polarized)	5.4	10.3
NC (noncomplementing)	20.7	34.9
Unknown	0	2.4
Total *ad-3* mutants	100.0 (92)[b]	100.0 (126)[b]
Leaky	30.4	10.1
Nonleaky	69.6	89.9
Total *ad-3* mutants	100.0 (92)[b]	100.0 (126)[b]

[a]No data available (see text).

[b]The total number of mutants analyzed.

higher percentage of the mutations induced by DEN + O_2 result in single amino acid substitutions in the polypeptide chains specified than those mutations induced by EMS. Due to the lack of information at the present time on other compounds, for example, methylating compounds with potent and weak carcinogenic activity, it is difficult to correlate this observation with the carcinogenesis of DEN.

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22.16 INHALATION CARCINOGENESIS

D. G. Doherty — Alfred Hellman
J. O. Brick — Paul Nettesheim
M. G. Hanna, Jr. — R. F. Newell

Previous reports[1] have fully described the protocol and experimental procedures used in setting up the inhalation chamber program. The current status of the chamber operations is summarized in Table 22.16.1. At this time three groups of animals in the inhalation chambers have reached a year or more total exposure to chamber conditions, and a few differences are beginning to emerge. The death rate is higher for those animals living in wire cages whether they are subjected to chamber exposure or not when compared with similar pretreated groups living in plastic pans. This seems primarily due to the increased incidence of urogenital and gastrointestinal disease and dermatitis in the animals on wire (Table 22.16.2). There also seems to be a trend towards urogenital disease in the animals exposed to chromic oxide dust and towards a low incidence of gastrointestinal disease in all the virus-treated groups. Sections of lungs of animals exposed to dust clearly show chromic oxide in the alveolar macrophages and the desquamated macrophages in the bronchus. Changes are also being seen in the lungs of animals exposed to the gasoline smog. Hair loss is more severe and occurs to a greater extent in all the animals living on wire than those controls in the plastic pans. So far no demonstrable pathology, bacteriology, or parasitology of the skin of affected animals can be seen. This is apparently a stress response brought about by the conditions of chamber living or living on wire. Routine monitoring of the chamber animals and those in pans has revealed no significant differences, all animals remaining free of internal and external parasites, pseudomonas, and salmonella and the nine viruses that can be typed for at present.

Table 22.16.1. Chamber Operations

Chamber Number	Pretreatment	Chemical	Exposure (hr)
I	½ irradiated	0	1399
II	½ irradiated	Cr_2O_3, 53 ppm 27 ppm	1243 363
III	½ irradiated	Ozone, 1 ppm; gasoline, 30 ppm	1500
IV	½ irradiated – all virus	Ozone, 1 ppm; gasoline, 30 ppm	1207
V	½ irradiated – all virus	Cr_2O_3, 53 ppm 27 ppm	723 363
VI	½ irradiated – all virus	0	993
VII[a]	0	45 pans	0
VIII[a]	Irradiated	45 pans	0
IX[a]	Virus	45 pans	0
X[a]	Irradiated – virus	45 pans	0
XI[b]	½ irradiated	45 pans	0
XII[b]	½ irradiated – virus	45 pans	0

[a]No exposure in the chambers – kept in pans to compare with chamber I and VI controls.

[b]No exposure in chambers – kept in wire cages to compare with chambers I, VI, VII, VIII, IX, and X controls.

Table 22.16.2. 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
Hydrocephalus	2		1	1	1		1	2				
Thymoma	1	2	3	1						1		
Starvation (long teeth)	3	4	2	3	1	2	2	3	2		2	2
Wasting (cause unknown)	3	5		1		1						
Physical injury (handling, etc.)	1	7	1			1	1					
Dehydration	2											
Urogenital disease	34	47	19	36	25	16	2	1	2		2	
Gastrointestinal disease		6	1	9	9	10			1	3	1	6
Respiratory disease	2	1	2	4	4	2	3		2	2		3
Leukemia	3	2	1	1			1	1		1	1	
Myocarditis			2		1		1	2	1	1		
Undetermined	21	48	18	13	10	8	7	18	7	11		7
Other (dermatis, etc.)	17	44	22	5	7	10	2	9	1	1	1	1
Total deaths in chambers	89	166	72	74	58	50	20	36	16	20	7	19

Four small inhalation chambers have been installed and calibrated. They are currently being used to determine the toxicity of a calcium chromate dust that is to be used in two additional large chambers that are currently being installed.

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22.17 REDUCED IMMUNE POTENTIAL OF AGED MICE: SIGNIFICANCE OF MORPHOLOGIC CHANGES IN LYMPHATIC TISSUE

M. G. Hanna, Jr. Paul Nettesheim
Takashi Makinodan[1]

Introduction. – Recent studies of the immunologic potential of aged mice by Makinodan and Peterson[2] have shown: (1) there is a decrease in the primary antibody-forming potential in 2.5- to 3-year-old mice, and (2) this is mainly due to a reduction in the number of potential antibody-forming cells. These results were obtained from intact animals as well as from the in vivo culture method in which a given number of spleen cells from donors of different ages are cultured together with the test antigen in heavily x-irradiated young adult isologous recipients.

Any study of the immune reaction in lymphatic tissue of aged animals is complicated by a number of specific and unspecific pathologic variables associated with aging. It was of importance to determine in connection with the studies of Makinodan and Peterson[2] whether the observed reduction in the number of potential antibody-forming cells is caused by specific pathologic changes in lymphatic tissue such as lymphoma or by more general changes associated with aging such as atrophy. It was also necessary to find out to what extent the spleen is representative of the immunologic capacity in the intact aged animal.

Methods. – Male and female BC_3F_1 mice 12 to 14 weeks and three years of age were used. The median life-span of both sexes is known to be ~138 weeks.

The first study was carried out in order to obtain preliminary information on the types and frequency of lesions likely to be encountered in three-year-old BC_3F_1 mice. Thirty-one mice were killed and autopsies were performed. All tissue samples were fixed in Bouin's fluid and stained with hematoxylin and eosin (H & E).

The second study was to determine the hemagglutinin response to 1 ml of 1% sheep erythrocytes (2×10^8 SRBC) in young adult (12 to 14 weeks of age) and three-year-old BC_3F_1 mice. Young adult and three-year-old mice were injected intravenously with 1 ml of 1% SRBC and groups of mice (between five and ten) were killed at days 1, 2, 3, 4, 6, 10, and 20 after injection. Along with serology, autopsies were performed on each animal.

The third study was to determine the response of splenectomized young adult and three-year-old mice to 1 ml of 1% and 10% sheep cells respectively. Blood was obtained by orbital bleeding one week prior to splenectomy to determine the level of natural SRBC hemagglutinin. Seven days after splenectomy, groups of young adult and three-year-old mice were intravenously injected with 1 ml of 1% and 10% SRBC respectively. On days 6, 10, and 15 after antigen, groups of splenectomized and nonsplenectomized young adult and three-year-old mice were killed. Blood was taken for serology, and autopsy was performed on each mouse.

Results and Discussion. – The results of the hemagglutinin response to test antigen (SRBC) in intact aged animals show a marked reduction in comparison with young adult mice. At both the 1% and 10% SRBC antigen doses the overall hemagglutinin response measured in the aged intact mice was less than 10% of the response in young adult animals. This reduction in primary antibody-forming capacity was determined in senile animals having no specific pathologic disorder of lymphatic tissue, suggesting that this is primarily due to unspecific atrophy or degenerative changes. Thus, this reduction in immune potential could well be a result of loss of progenitor cells or a decrease in the capacity of uncommitted immunologically competent cells due to preoccupation to other antigens, as is also indicated by the large number of mature plasma cells in the medulla of superficial lymph nodes.

The histopathology of the lymphatic tissue of "normal" non-antigen-stimulated old mice showed that atrophy and degenerative changes in spleen

Table 22.17.1. Hemagglutinin Production of Sheep Erythrocytes in Splenectomized Young Adult and Three-Year-Old BC_3F_1 Male Mice[a]

Treatment	Background Log_2 Titer	Log_2 Titer, 1 ml 1% SRBC			Log_2 Titer, 1 ml 10% SRBC		
		Day 6	Day 10	Day 15	Day 6	Day 10	Day 15
Young Adult Mice							
Nonsplenectomized	1.2 ± 0.3		10.0 ± 0.1 (10)	10.0 ± 0.2 (10)	10.0 ± 0.1 (10)	9.5 ± 0.1 (10)	9.5 ± 0.2 (10)
Splenectomized			5.0 ± 0.5 (10)	5.3 ± 0.5 (10)	5.1 ± 0.6 (9)	6.3 ± 0.8 (10)	5.6 ± 0.4 (9)
Aged Mice							
Nonsplenectomized	1.7 ± 0.7		8.3 ± 0.3 (6)	3.3 ± 0.4 (7)	6.3 ± 1.0 (9)	6.0 ± 1.0 (8)	6.3 ± 0.9 (9)
Splenectomized			1.6 ± 0.5 (7)	2.3 ± 0.3 (2)	2.3 ± 0.4 (8)	2.9 ± 0.6 (7)	3.7 ± 0.6 (7)

[a]Number of mice used at each point in parenthesis. Each mean presented with one standard error.

lymphoid tissue were less pronounced than in other nonsplenic lymphatic tissue. This suggested that the spleen was the major source of immunological competence in the intact aged animals. The results of splenectomy (Table 22.17.1) showed that with the lower antigen dose, loss of splenic lymphatic tissue completely eliminates any measurable immunological potential in these aged mice, while with the higher antigen dose there was a weak hemagglutinin response from nonsplenic sites which was only threefold higher ($\log_2$ titer) than background and 12.0% of that achieved in the splenectomized young adult mice. This promotes the idea that in mice of this age group the spleen is representative of the immune capacity of the intact animal, but that it is, however, not necessarily representative of the immune potential of the other lymphoid organs.

A major limitation of this study is that we were working with a very select group of animals, that is, those that survived to three years of age. The complexities of the physiologic and pathologic states of these aged mice also complicate the interpretations of the present studies. However, the importance for further systematic studies of this type in order to establish a base line for the old animal is clear. From these parameters we might better understand the changing incidence of diseases which accompany old age. Also, the role of environmental factors during aging can be more readily evaluated once the physiologic and pathologic states of the aged animals are more clearly defined.

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22.18 SUBCELLULAR FRACTIONATION STUDIES OF ^{125}I-SOLUBLE ANTIGEN RETENTION IN SPLEEN LYMPHATIC TISSUE AND LIVER

Mary W. Francis M. G. Hanna, Jr.
W. D. Fisher[1]

The early localization of intravenously injected soluble antigen in the spleen germinal centers reported by Hanna *et al.*[2] has posed the question of the form of the antigen at this site. This localization precedes measurable serum titer, but it has been associated with a definite reaction by the host to the antigen at a later time.

Garvey and Campbell[3] and Ada and Lang[4] have both studied the form of the antigen being retained in the liver and in the lymph node. Their experiments determined that soluble antigens (BSA and HSA) were associated with the soluble portions of the cell. These investigations were not mainly concerned with the spleen during early intervals of the primary response.

The following is a series of preliminary experiments in primary stimulated mice designed to determine the location of the antigen within the cell and to evaluate the lability of the ^{125}I antigen under the conditions of the experiments.

Methods. – Eleven-week-old BC_3F_1 male mice were injected intravenously with ^{125}I-labeled human gamma globulin (30 μc/animal). Spleens, livers, and sera were collected 2 hr, 1, 4, 6, 7, 15, and 18 days after the injection. Homogenates (10%) of liver and spleen were made in 0.25 *M* sucrose containing 0.01 *M* Tris buffer and 0.01 *M* $CaCl_2$. Homogenization was accomplished with a Teflon-pestle Potter-Elvehem motor-driven homogenizer. Microscopic examination of the homogenates confirmed cellular disruption.

The homogenates were layered (0.3 ml) onto individual sucrose gradient tubes (15 to 55% sucrose with 2.0 ml of 66% sucrose as a cushion, all made with 0.01 *M* Tris and 0.01 *M* $CaCl_2$). A 1.5-ml overlay of 0.01 *M* Tris was placed over the homogenate. The tubes were placed in the SW-25 rotor and spun at 20,000 rpm for 2 hr in the Beckman model L centrifuge. The gradients were pumped from the bottom of the gradient tubes and monitored for protein in a 0.2-mm flow cell in the Beckman DB. Fractions were collected in 15-drop aliquots and counted in the Packard Autogamma counter.

The fractions were divided roughly into nuclei and cellular debris, mitochondria and large particles, and the "soluble" portions. The soluble fractions of days 4, 6, and 7 were pooled and spun at 105×10^3 *g*'s for 5 hr. The supernatants and precipitates from these fractions were separated, and specific activity was determined by Autogamma counting and protein analysis, using a modification of the Lowry method for protein determination (results expressed as counts per microgram of pro-

tein). There are "particles" in the soluble portion of this division, as shown by the precipitation with rigorous centrifugation. The specific activity of the supernatant of this process is nondialyzable, as shown by the specific activity after dialysis.

To determine how labile the ^{125}I-HGG was in these supernatant fractions they were dialyzed at 5°C against 0.01 *M* Tris buffer at pH 7.4 and then concentrated with polyglycol. Specific activity was again determined. A trichloroacetic acid precipitation on the whole "pools" was used to estimate the total amount of nonassociated label. Three washes of the precipitates were found to yield only a few more counts than the original supernatant.

After concentrating the dialyzed pools, paper electrophoresis at pH 8.6 was used to assess the nature of the protein/proteins with the label. The paper strips were cut into bands after being monitored on the Beckman Analytrol, and the bands were counted.

Results and Discussion. – The separation of large particles (nuclei and cellular debris), small particles (mitochondria and large granules), and cell "soluble" portions was made on the basis of the monitored record and microscopic examination. It is recognized that this is only a rough division, but it is sufficient for these preliminary experiments.

In both spleens and livers, at 24 hr ~60% of the label recovered was in the "soluble" cut, with ~30 and 10% in the large- and small-particle cut respectively. Between days 7 and 15 the "soluble" cut remained constant between 50 and 60% recovered activity, while a shift in the activity was measured between the large- and small-particle cuts (measured activity was ~15 and 34%, respectively, at day 15).

Electrophoretic analysis of these dialyzed pools indicated that the label is primarily associated with a protein having the same mobility as that of serum gamma globulin. It is as yet undetermined whether this labeled protein is associated with the mouse globulin or is free human gamma globulin.

Further protein separations are planned which, along with determinations of the antigenic and physical properties of the labeled protein, may yield information of the nature of the antigen associated with the germinal center at the later intervals of the immune response.

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22.19 ULTRASTRUCTURAL STUDIES OF ANTIGEN LOCALIZATION IN SPLEEN LYMPHATIC TISSUE

A. K. Szakal M. G. Hanna, Jr.

Introduction. – Recent experiments of spleen lymphatic tissue in mice intravenously injected with ^{125}I-labeled isologous and heterologous macroglobulins have shown by light-microscopic autoradiography the retention and localization of heterologous soluble antigen in the germinal centers.[1] To supplement this finding electron-microscopic autoradiography was used to more accurately define the cell types in lymphatic tissue germinal centers involved in the retention of antigen. It was also expected that this technique would demonstrate the intracellular sites of antigen localization.

Methods. – Young adult BC_3F_1 mice were intravenously injected with 1 ml of 1.5 mg/ml of ^{125}I-labeled human gamma globulin (HGG) having a specific activity of 20 μc/mg. The animals were sacrificed at 2 hr and at 1, 2, 5, 10, 15, and 20 days after the time of injection. After removal the spleen was bisected longitudinally, and the two halves were fixed in 1% glutaraldehyde in three-fourths-strength Tyrode's solution at pH 7.2 for ½ hr to enhance the dissection of individual lymph nodules. Following the dissection of lymph nodules the fixation in glutaraldehyde was continued for another 1½ hr. Postfixation was accomplished in pH 7.2 phosphate-buffered osmium tetroxide. Subsequently, the tissues were embedded in epoxy resin. The individual germinal centers were localized on 1-μ-thick sections stained with a mixture of methylene blue and azure II. After locating the germinal center, ultrathin sections were cut for electron-microscopic

autoradiography. The sections were stained with uranyl acetate and lead citrate and prepared for autoradiography according to the techniques described by Caro[2] using Ilford L-4 nuclear research emulsion. The grids were then stored in the dark for eight to nine weeks to attain proper exposure of the emulsion. After development of the specimens in Kodak Microdol-X the sections were examined by an HU11B Hitachi electron microscope.

Results. – Uptake of antigen, as represented by silver grains of the microautoradiographs in red pulp, lymphatic nodules, and germinal centers, was most prominent in the cells of the germinal center and, to a lesser degree, in the surrounding white pulp. The red pulp did not show any appreciable concentration of label in any of its cell types. In animals killed after 2 hr and one and two days, small numbers of silver grains indicating the presence of HGG were apparent extracellularly and attached to erythrocytes in the red pulp. In the white pulp silver grains were in the cytoplasm of small- and medium-size lymphocytes among the numerous free ribosomes (Fig. 22.19.1*A*) and in a small fraction of macrophages containing cellular debris in their cytoplasm. An increased number of silver grains were found in cells of the germinal center in animals sacrificed at later intervals. Based on ultrastructural morphology these cells were distinguished as large lymphocytes, plasmablasts, and plasmacytes. In these cell types silver grains were located in some of the mitochondria and in close association with the cisternae of the endoplasmic reticulum (Fig. 22.19.1*B*).

The results of this preliminary experiment do not permit any interpretation or elaboration on the possible significance of the location of silver grains in germinal center cells. These findings need confirmation by a similar experiment presently in progress.

References

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22.20 FACTORS INFLUENCING THE X-RAY RECOVERY OF SECONDARY ANTIBODY-FORMING POTENTIAL

Paul Nettesheim Mary Lou Williams

Introduction. – Previous studies in our laboratory have been concerned with the recovery potential of immunologically competent progenitor cells from x irradiation. We were able to show that the compartment of immunologically uncommitted progenitor cells (PC_1), that is, the primary antibody-forming potential, has the capacity to recover from an x-ray insult. In contrast, the compartment of immunologically committed progenitor cells (PC_2), that is, the secondary antibody-forming potential, was shown to lack the capacity to recover from x irradiation under the conditions tested (mice were primed with $\sim 10^8$ rat red blood cells four weeks before irradiation). Preliminary experiments indicated, however, that altering the amount of antigen given as primary injection or the time interval between priming and x irradiation did have some effect on the secondary-response recovery.

Materials and Methods. – Therefore, eight-week-old male and female C31F1 mice were primed intraperitoneally with rat red blood cells (rbc) as test antigen, varying the dose over a 1000-fold range (10^7 to 10^{10} rat rbc). Four weeks later they were irradiated with either 0 or 400 r. Their spleen cells were then tested various times after x irradiation for antibody-forming potential using the in vivo culture technique (i.e., a fixed number of spleen cells is mixed with optimum amounts of antigen, injected into lethally irradiated recipients, and the antibody produced on day 8 after culture is determined).

Results and Discussion. – The experiments are summarized in Fig. 22.20.1, in which the antibody-forming potential of irradiated spleen cells is expressed as percent of the 0-r controls. The initial x-ray suppression of the antibody-forming potential amounted to $\sim 90\%$ regardless of the priming dose. Definite though incomplete recovery was seen only in the groups receiving 10^9 and 10^{10} rat rbc as priming dose. However, no recovery was observed in the groups receiving 10^7 and 10^8 rat rbc, as described previously. In subsequent experiments mice were primed with 10^8 rat rbc and were irradiated two weeks later (rather than four weeks). Rapid recovery occurred during the first

PHOTO 86870

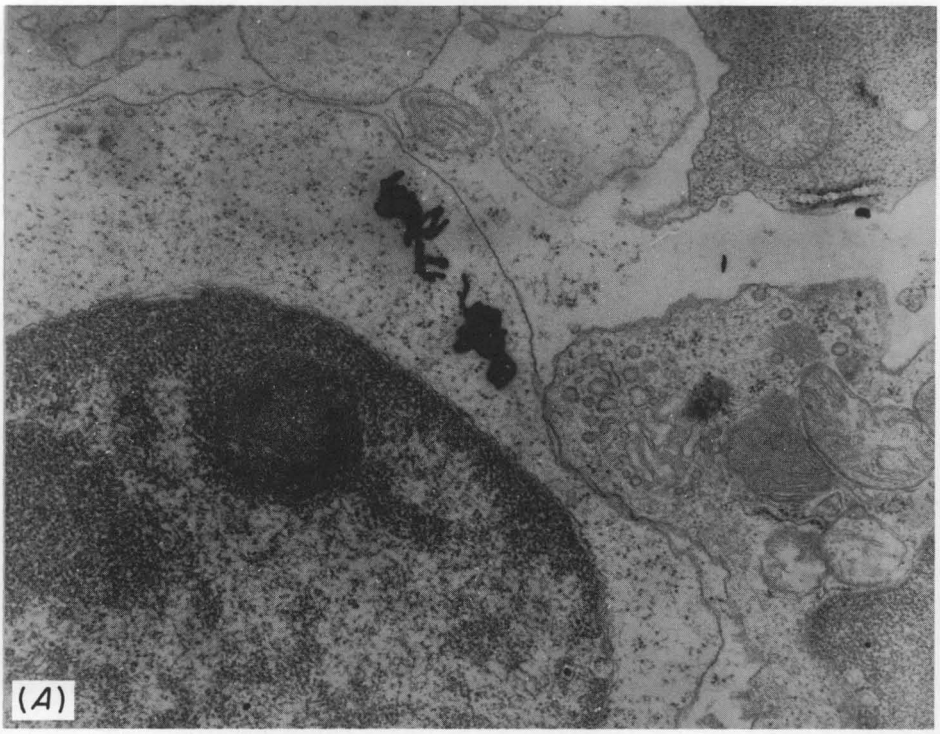

Fig. 22.19.1. Electron Microscope Autoradiograph, Showing Localization of ^{125}I-Labeled HGG in the Cytoplasm of a Small Lymphocyte (*A*), 22,500×, and Plasmablast (*B*), 30,187×.

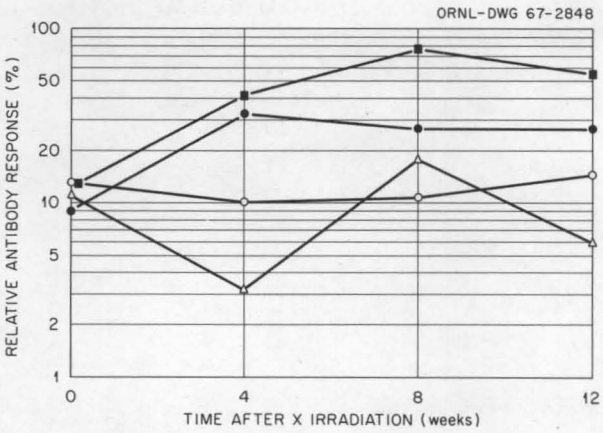

Fig. 22.20.1. Secondary Antibody-Forming Potential of 25×10^6 Spleen Cells with Time After Irradiation with 400 r. Spleen cells from donors primed with 10^7 rat rbc (Δ), 10^8 rat rbc (o), 10^9 rat rbc (●), and 10^{10} rat rbc (■). Ten to fifteen samples per point.

four weeks after irradiation. These data confirm and extend our previous results. They indicate that the PC_2 cell compartment does not recover after an x-ray insult unless antigen is persisting at the time of x irradiation in amounts large enough to prime PC_1 cells – which are known to recover after irradiation – thereby refilling the PC_2 cell pool. Studies are under way to clarify the mechanism of secondary-response recovery occurring under the conditions described.

22.21 AMINOACYL sRNA LEVELS IN VIRAL AND NONVIRAL INFECTED MAMMALIAN CELLS

Alfred Hellman L. J. Wopschall
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Alteration in sRNA and aminoacyl sRNA synthetase levels of phage-infected *E. coli* were previously reported. This suggested to us an investigation of the influence of vaccinia virus and polio virus infection on the levels of aminoacyl sRNA synthetases in HeLa cells propagated in suspension culture. Similarly we determined the temperature sensitivity of these synthetases when exposed to 50°C for 10 min. We have quantitatively investigated the charging activity of the normal and viral-infected HeLa cells for 15 amino acids. Possible quantitative changes in synthetase levels and alteration in temperature sensitivity of HeLa cells propagated at 40°C rather than at our customary 34°C incubation temperature are also being investigated. The rationale for the 40°C incubation was the apparent genetic variability of the attenuated and virulent polio viruses, the latter being able to be propagated at 40°C. We have observed temperature stability of threonyl sRNA synthetase and a threefold increase in charging activity of glycyl sRNA synthetase after exposure of the enzyme to 50°C for 10 min. However, we could not distinguish any significant differences in enzyme levels between the infected and noninfected cell population.

22.22 IN VIVO PROTEIN DAMAGE TO *ESCHERICHIA COLI* BY BENZO(a)PYRENE WITH 355 mμ ILLUMINATION

A. P. Harrison, Jr. Vivian E. Raabe

In vitro photodynamic inactivation of protein with methylene blue involves a preferential photooxidation of the aromatic amino acids and histidine.[1,2] (Aliphatic amino acids such as leucine are not attacked.) Preferential attack of these amino acid moieties in vivo has not been described.

We have wished to be able to monitor in vivo protein damage during illumination with photosensitizer. The research reported here was undertaken to devise a method for this and also to determine whether the amino acids of intracellular protein exhibit the same relative susceptibility to photodynamic attack as described in vitro.

Preliminary work has employed *Escherichia coli* 15H–T–L–, a triple auxotroph requiring histidine, thymine, and leucine. Illumination was with a pair of G.E. BLB "blacklight" fluorescent tubes emitting symmetrically about 355 mμ and at a dose rate of 80 ergs mm^{-2} sec^{-1}. Benzo(a)pyrene was photosensitizer. It was administered as a colloid at a concentration of 1 μg/ml in 0.03 *M* sodium phosphate buffer.

One batch of cells was grown in uniformly labeled ^{3}H-L-histidine. A second batch of cells was grown in ^{3}H-L-leucine. We assume that photodynamic damage to a tritiated amino acid will cause loss of tritium from that amino acid into the medium; thus the amount of radioactivity

in the medium will reflect the degree of protein damage. The composition of the cultivation medium and the amount of label were carefully adjusted so that each batch of cells incorporated the respective amino acid at identical specific activities.

When a culture had attained a concentration of 5×10^8 cells/ml, incorporation of radioactive label was diminished by adding a 100-fold excess of unlabeled amino acid, and the culture was permitted one further doubling. The log-phase cells were then harvested and were washed three times at the centrifuge. The cells were resuspended in phosphate buffer with and without benzo(a)pyrene. Ten-milliliter cell suspensions were dispensed into three small petri dishes. One dish containing benzo(a)pyrene was illuminated, another was held in darkness (dark control). A dish lacking benzo(a)pyrene was illuminated (illumination control). Histidine- and leucine-labeled cells were tested simultaneously; thus an experiment comprised a total of six petri dishes. The temperature was held at 6°C by means of ice surrounding the petri dishes. Periodically, a 1-ml sample was withdrawn from each dish, was centrifuged, and the cell-free supernatants were assayed for radioactivity.

Negligible radioactivity was detected from the dark and illumination controls with either ^{3}H-histidine- or ^{3}H-leucine-labeled cells. On the other hand, ^{3}H-histidine-labeled cells in the illuminated dish with benzo(a)pyrene released radioactivity into the medium; the ^{3}H-leucine-labeled cells did not. In replicate experiments with higher specific radioactivities some release of label occurred from both ^{3}H-histidine- and ^{3}H-leucine-labeled cells in the illuminated dishes with hydrocarbon, but always the loss of label from the former was much greater (6 to 8 times greater) than that from the latter. These experiments, as already noted, were carried out at 6°C. When they were carried out at 34°C the rate of tritium release from both classes of labeled cells in the illuminated dishes with hydrocarbon was about the same and was much greater than at 6°C.

The following interpretation is considered and is tentative. Preferential photooxidation of the histidine moieties of protein in *E. coli* may occur. But at metabolic temperatures additional reaction may also occur. Release of tritium from protein labeled with ^{3}H-histidine may serve as a convenient and sensitive indicator of in vivo photodynamic damage to *E. coli* protein.

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22.23 HORMONAL REGULATION OF ENZYME SYNTHESIS IN SUSPENSION CULTURES OF THE REUBER HEPATOMA

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Introduction. – In earlier studies with monolayer cultures of the Reuber hepatoma[2,3] it was found that the hormonal inducers of tyrosine transaminase in rat liver[4] (hydrocortisone, insulin, and glucagon) could also induce the enzyme in these neoplastic "mutants." These studies were complicated by the variation found to occur from one culture bottle to another. While this variation was not as large as that encountered in a population of living animals, it was large enough to complicate the experimentation and in this sense to negate the value of this in vitro test system. Methods were developed to permit the hormonal studies to be carried out in suspension cultures from which identical aliquots can be taken at different points in time.

Results and Discussion. – Addition of hydrocortisone (10^{-6} *M*) to suspension cultures elicited a six- to tenfold increase in tyrosine transaminase levels. The hydrocortisone response was maximal by 12 hr and remained at this elevated level as long as 24 hr. Insulin (10 milliunits/ml) or glucagon (10 μg/ml) initiated a doubling of the enzyme level within 6 hr following hormone addition. Thereafter, the enzyme level gradually declined and by 24 hr had reached control levels. From the results presented here it is clear that suspension culture of the Reuber hepatoma offers a convenient in vitro system in which to study hormonal control of enzyme synthesis in this tumor without the masking influence of the in vivo environment.

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22.24 TYROSINE TRANSAMINASE IN REGENERATING LIVER

Claudio Pellegrino F. T. Kenney

Introduction. – Previous work has shown that the level of tyrosine transaminase in fetal rat liver is less than 10% of that in the adult at all stages of gestation and that the enzyme is not inducible.[1] Subsequently it was seen that decapitation of the fetus permits a moderate degree of induction, suggesting that pituitary factors are implicated in the repression of the synthesis of this enzyme.[2] It was thought that a study of regeneration of the adult liver after partial hepatectomy, in which the liver cells undergo rapid multiplication, might give useful elements for a comparison with the fetal situation. This would enable us to determine if impairment of regulatory responses is characteristic of growing tissues.

Results and Discussion. – The results obtained so far show that levels of tyrosine transaminase are elevated above the normal in rats partially hepatectomized 24 and 48 hr earlier and with intact adrenals. Hydrocortisone administered to adrenalectomized animals partially hepatectomized 24 hr earlier gave enzyme induction comparable with that obtained in adrenalectomized, sham-operated rats.

It is thus clear that regenerating liver at 24 hr after operation (a time interval at which the cells are about to undergo mitosis) shows no impairment in tyrosine transaminase induction by hydrocortisone. A difference with the behavior of tryptophan pyrrolase, which, according to recent reports,[3,4] is less inducible by hydrocortisone at this same time interval after hepatectomy, is apparent.

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22.25 REGULATION OF ENZYME SYNTHESIS BY HYDROCORTISONE IN ORGAN CULTURES OF FETAL RAT LIVER

W. D. Wicks[1] J. D. Regan[2]

Introduction. – A previous report described attempts undertaken to induce tyrosine transaminase in fetal rat liver in utero by hydrocortisone.[3] Earlier workers had been unable to increase the activity of this enzyme with a variety of agents known to induce the adult enzyme.[4] Hypophysectomy of fetuses allowed hormonal induction in a few cases, but extreme variations in response prompted a shift in experimental procedure. It was decided to maintain fetal liver in organ culture in an effort to remove (and perhaps identify) potential factors which interfered with enzyme induction.

Results. – Livers from 16- to 22-day fetuses were cut into 2-mm^3 cubes and placed on stainless steel grids in tissue culture dishes. The livers were kept in contact with a medium composed of Eagle's minimal essential medium (76%), tryptose phosphate broth (14%), and calf serum (10%). The cultures were kept at 37° in a humidified atmosphere composed of 98% air and 2% CO_2.

Histological examination revealed that hepatocytes retained a normal appearance for at least three days in culture except for the loss of their glycogen deposit. The incorporation of [^{14}C]-valine into protein also remained constant during this period.

During the first 12 hr in culture no change in transaminase activity was produced by the addition of 10^{-5} to 10^{-7} *M* hydrocortisone. Thereafter, however, and up to at least 72 hr, reproducible fivefold increases in enzyme activity were achieved with 10^{-7} *M* hormone. The specific activity of the enzyme depends upon both the age of the donor fetuses and the time in culture. The level of activity increased slightly during gestation and rose slowly during the period in culture. In spite of this the extent of induction, usually fivefold, and

the time course of induction were identical with livers from donors of all ages and at any hour in culture (up to 72). The maximum response to hormone was usually attained within 5 to 10 hr, and the induced level was maintained for at least 24 hr. If hormone is removed by changing the medium, the elevated level was seen to fall in a preliminary experiment after a lag of 2 to 3 hr.

Aims of future experiments are, among others, determining whether de novo synthesis of the enzyme is involved in the hormonal response, and estimating the half-life of the enzyme immunochemically.

Discussion. – These results clearly show that the fetal liver possesses the biochemical machinery required to respond to hydrocortisone by an elevation of tyrosine transaminase activity in spite of the very low activity and essential noninducibility of this enzyme in utero. The results suggest further that certain agents may be circulating in the fetal environment which act to restrain the activity of this enzyme until birth. Attempts will be made to locate and identify these as yet hypothetical agents.

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AEC-NASA SPACE BIOLOGY (22.26 – 22.29)

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22.26 THE *NEUROSPORA* EXPERIMENT ON BIOSATELLITE A

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The mission of the Biosatellite program is to investigate the interaction of living organisms with certain aspects of the space environment. The three-day flights were designed primarily to study (1) the effects of weightlessness on basic biological processes and (2) the effects of irradiation under weightlessness on such genetic effects as killing, mutation induction, and production of chromosome rearrangements. In the three-day flights the spacecraft has a built-in radiation source (^{85}Sr) to make it possible to obtain known doses of radiation so that dose-effect curves for various genetic effects can be obtained under "weightlessness."

The *Neurospora* experiment utilizes conidia of a genetically marked two-component heterokaryon collected on the surface of Millipore filters in a sealed package. The experiment is designed to study (1) inactivation of the heterokaryotic conidia which with ionizing radiation results from one-hit events believed to be terminal chromosome deletions and (2) gene mutation resulting from both one- and two-hit events. The type of one-hit event resulting in mutation is qualitatively different from the one-hit event resulting in cell inactivation. The two-hit mutations result from chromosome deletion and are expected to respond to changes in environmental conditions in the same way as the one-hit events resulting in cell inactivation.

In the Biosatellite the *Neurospora* samples obtain an almost continuous array of gamma-ray exposures ranging from about 500 to 6000 r in the flight vehicle as well as in a control experiment on the ground with an identical radiation source in a simulated spacecraft. Samples were also placed in the shielded sections of both vehicles to serve as unirradiated controls. Samples of conidia are

analyzed by the direct method to obtain dose-response curves for survival and induction of purple adenine mutants resulting from mutation in the *ad-3* region.

The flight of Biosatellite A took place in mid-December 1966. The spacecraft was launched successfully on December 14, 1966, from Cape Kennedy, and the telemetry data indicated that the radiation source holder opened approximately 1 hr after launch and closed about 65 hr later on schedule. However, the retrorocket failed to operate at time for deorbit, so that the satellite and its payload remained in orbit.

Since the in-flight samples were not recovered, only the samples from the ground control experiment were analyzed. The dose-response curves are in excellent agreement with other experiments with ^{85}Sr and ^{137}Cs gamma rays at the same dose rate and with 250-kv x rays at 10 r/min. Nevertheless, the first attempt at flight provided the only set of control data obtained with flight hardware, which will provide important control data for evaluation of data from in-flight samples from the flight of Biosatellite B, tentatively scheduled for mid-April 1967.

22.27 THE FLIGHT OF BIOSATELLITE A

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The first Biosatellite was launched from Cape Kennedy at 1420 hr on December 14, 1966. When signaled to reenter the earth's atmosphere on December 17, 1966, the retrorocket did not fire. Biosatellite A is still in orbit, and recovery of the dead Habrobracon from it is not expected. The ground-based control for the experiment is being carried through to completion. Further details on the planning and execution of the Habrobracon experiment in the Biosatellite Project are given elsewhere.[2–4]

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22.28 HABROBRACON SURVIVAL AND DOMINANT LETHALITY IN THE GROUND-BASED CONTROL FOR THE 301 WALK-THRU TEST OF THE BIOSATELLITE EXPERIMENT

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Introduction. – The purpose of this experiment was to obtain data during a complete dress rehearsal on the backup (301) Biosatellite vehicle. Following the test we examined Habrobracon for their survival and measured dominant lethality induced by ^{85}Sr over a $4\frac{1}{2}$-day period. This was to mimic the conditions of the actual flight. The analysis was carried out only on animals used in the ground-based control, since only this part of the experiment was carried through to completion. The 301 flight vehicle was not used beyond the time of the artificially scheduled launch.

Results. – Table 22.28.1 summarizes the survival of lemon males within each module position. The higher survival in the 4000- and 2000-r nominal dose positions was undoubtedly because the youngest males were placed in these modules. Low survival values occurred in the center layer of each module of the controls. This was probably due to the crowding effect on the center layer by the outer and inner layers. This effect was eliminated in subsequent tests by placing younger animals in each layer. The lemon males from each dose level were mated to virgin females of the Raleigh strain. Eggs were counted, and hatchability was recorded. Dominant lethality in terms of diploid embryo survival is summarized in Table 22.28.2. The percent fertilization and frequency of induced dominant lethality were computed according to the method of von Borstel and Rekemeyer.[1]

Discussion. – This is the fourth test in which both survival and dominant lethality measurements were taken under conditions simulating the actual

Table 22.28.1. Survival of Lemon Males in 301 Walk-Thru Test

Nominal Dose (r)	Module Position	Number of Wasps	Number of Survivors	Percent Survival
4000	UL	20	20	100
	LR	20	20	100
2000	UL	20	20	100
	LR	20	20	100
1000	UL	20	16	80
	LR	20	19	95
500	UL	20	17	85
	LR	20	16	80
Control				
Inner	UL	20	17	85
Middle		20	9	45
Outer		20	17	85
Inner	LR	20	16	80
Middle		20	9	45
Outer		20	18	90

Table 22.28.2. Diploid Embryo Survival in 301 Walk-Thru Test

Nominal Dose (r)	Measured Dose (r)	Number of Eggs	Diploid Embryo Survival
0	2.6	357	0.939
500	492	1001	0.822
1000	1070	959	0.650
2000	1920	1028	0.498
4000	3440	1053	0.235

radiation conditions of the flight. The data are in accord with the previous tests.[2–4]

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22.29 THE GEMINI XI S-4 EXPERIMENT. II. THE NEUROSPORA EXPERIMENT

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M. A Bender

In the S-4 blood experiment on Gemini III an apparent synergism between space-flight conditions and radiation was found which resulted in a significantly greater rate of single-break chromosome aberrations.[3] The S-4 blood experiment was repeated on the Gemini XI mission. A *Neurospora* experiment was also flown on Gemini XI to permit an independent evaluation of the genetic effects of irradiation during space flight. This would make it possible to determine whether the apparent synergism on the Gemini III mission was the result of a general synergism between "weightlessness" and radiation. The design of the *Neurospora* experiment, however, also made possible a completely independent evaluation of the genetic effects of radiation and space-flight parameters both on chromosome breakage and production of gene mutations at specific loci.

The apparent synergism (a significantly greater rate of single-break chromosome aberration production) observed in the S-4 blood experiment on Gemini III should be reflected in lower levels of survival and higher frequencies of gene mutation. Conidia (from a genetically marked two-component heterokaryon) were collected on the surface of Millipore filters (standard procedure) or in suspension (to mimic the S-4 blood experiment) and simultaneously irradiated with different exposures of ^{32}P beta rays both on the ground and in flight during the orbital phase of the Gemini XI mission. The same device that was designed for the S-4 blood experiment on Gemini III was used for the S-4 *Neurospora* experiment.

After launch of Gemini XI the in-flight experimental device remained essentially "weightless" during all the irradiation period (approximately 38 hr) except for the two Agena PPS burns (which each lasted approximately 22 sec and produced

an acceleration of about 1.2*g*). The in-flight samples remained "weightless" until about 3 ½ hr prior to retrofire. On recovery of the irradiation device from the spacecraft, the *Neurospora* samples were removed from both the in-flight and ground devices, refrigerated, and returned to ORNL for genetic analysis. The samples were thus subjected to three days postirradiation storage prior to analysis.

Results. – The analysis of the dose-effect curves for survival and forward mutation for all the in-flight and ground control samples has been completed. The more detailed genetic analysis of the *ad-3* mutations in each sample is in progress.

The data from the analysis of samples on filters show no significant difference in survival curves (Fig. 22.29.1) or forward-mutation curves (Fig. 22.29.2). The data from the samples in suspension show that a significantly higher ($P = 0.02$) survival was obtained with the in-flight samples than with the ground control samples. There is no difference in the slopes of the dose-effect curves for forward mutation, and if we assume that they are identical, then covariance analysis of these data indicates a highly significant difference between these two curves ($F = 236.7$ with one and five degrees of freedom).

The difference in the dose-effect curves for mutation between the filter samples and those in suspension can be attributed primarily to the protection obtained by irradiation under conditions of anoxia. Anoxia gives higher levels of survival if the conidia are plated immediately; the difference

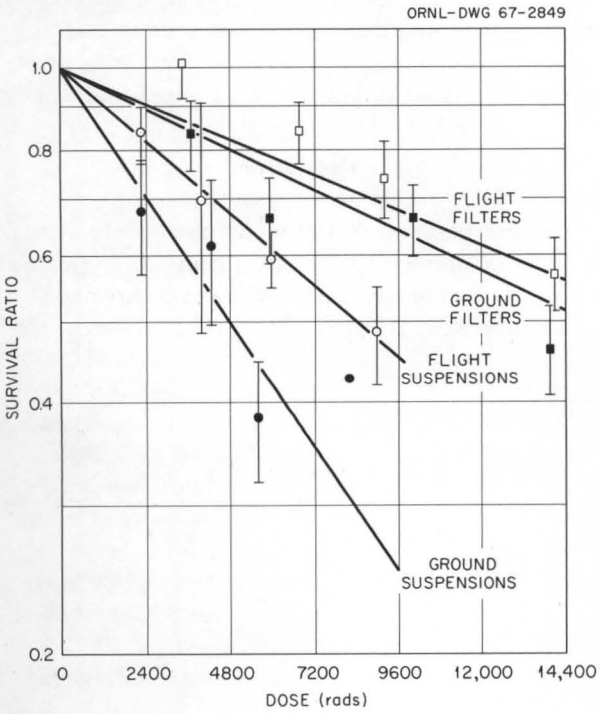

Fig. 22.29.1. Dose-Effect Curves for Survival for Flight and Ground Samples of the S-4 Neurospora Samples. □ = flight filter, ■ = ground filter, O = flight suspension, ● = ground suspension.

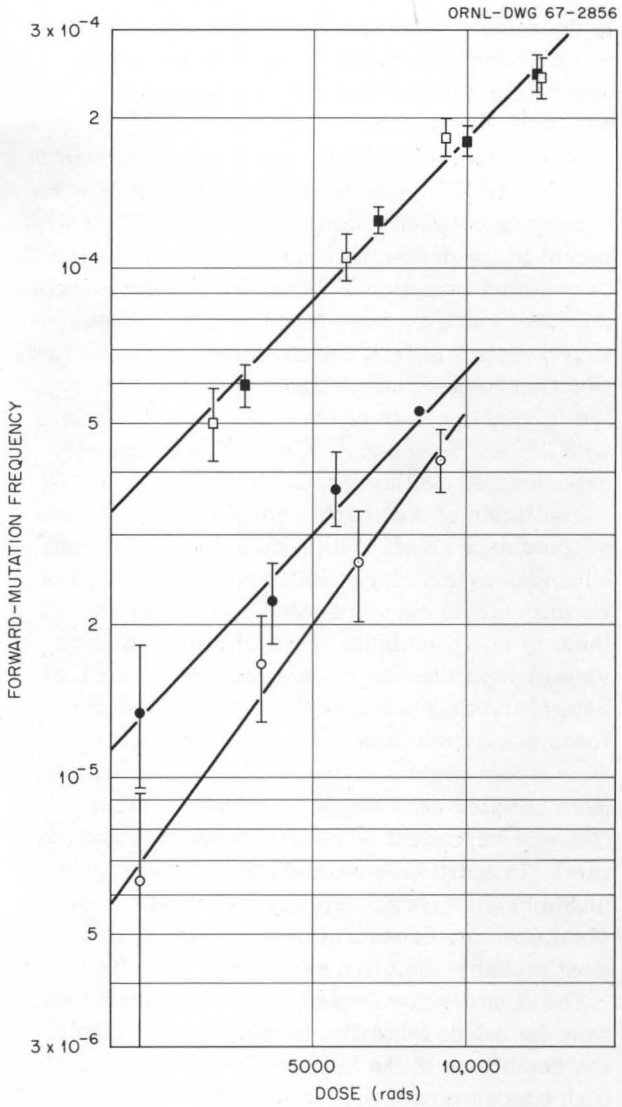

Fig. 22.29.2. Dose-Effect Curves for Forward Mutation in the *ad-3* Region for Flight and Ground Samples on the S-4 Neurospora Samples. □ = flight filter, ■ = ground filter, O = flight suspension, ● = ground suspension.

in the effects observed, from those expected, can be attributed to postirradiation storage, which affects the survival of conidia in suspension but not conidia on filters.

Discussion. – The data from the S-4 *Neurospora* samples on Millipore filters show that there is no synergism between radiation and space flight with regard to the dose-effect curves for inactivation or mutation induction and that the genetic effects are identical with those found in ground-based experiments. In fact, the estimates of the forward-mutation frequencies obtained with ^{32}P beta rays are in excellent agreement with those obtained with 250-kv x-ray and ^{137}Cs or ^{85}Sr gamma-ray exposures at similar dose rates.

Irradiation of *Neurospora* conidia on filters was adopted as a standard procedure for experiments with various ionizing radiations because (1) they remain aerobic even in sealed containers and (2) there is no appreciable effect of postirradiation storage (up to ten days) on either the survival of heterokaryotic conidia or the radiation-induced forward-mutation frequency. The irradiation of *Neurospora* conidia in suspension, however, is more complex because they become anaerobic (the rate dependent on concentration and temperature). In addition, postirradiation storage under anaerobiosis gives a very rapid decrease in survival (the rate dependent on temperature, and most probably radiation exposure).

The *Neurospora* samples flown as suspensions were included, primarily, to mimic more closely the conditions of the S-4 blood experiment. A high concentration of conidia was used so that the suspensions would become anaerobic rapidly (within 75 min at 25°C) and remain anaerobic throughout the irradiation period. The main difference expected between the dose-effect curves for conidia on filters and those in suspension is the protection obtained by irradiating under conditions of anoxia (higher survival levels and lower forward-mutation frequencies); but the dose-effect curves are modified (decrease in survival) by the prolonged (three days) postirradiation storage at 4°C. Thus, the radiation response of *Neurospora* conidia on Millipore filters is essentially temperature-independent, while that of conidia in suspension is markedly temperature-dependent. We have concluded, therefore, that the difference in the dose-effect curves for the in-flight and ground control suspensions reflects unintentional differences in the physiology of these two sets of samples (due to unavoidable differences in in-flight and ground device temperatures) rather than evidence for antagonism between radiation and some space-flight parameter. In summary, the S-4 *Neurospora* experiment, like the S-4 blood experiment on Gemini XI, (1) failed to confirm the apparent synergism seen in the Gemini III S-4 blood experiment and (2) indicated that there is no difference in the genetic effects of irradiation during space flight from the genetic effects obtained in ground-based experiments.

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246. Paul Kotin, National Cancer Institute, Bethesda
247. Russell S. Poor, Atomic Energy Commission, Washington
248. Robert Rabson, Atomic Energy Commission, Washington
249. H. M. Roth, Research and Development Division, AEC, ORO
250. M. B. Russell, University of Illinois, Urbana
251. C. S. Shoup, Atomic Energy Commission, Oak Ridge
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254. C. G. Zubrod, National Cancer Institute, Bethesda
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258–261. Atomic Energy Commission, Washington
262–265. Battelle Northwest
266–267. New York Operations Office
268. Patent Branch, Washington
269–272. Division of Technical Information Extension, Oak Ridge