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Progress

BIOLOGY DIVISION
SEMIANNUAL PROGRESS REPORT
FOR PERIOD ENDING AUGUST 15, 1963

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ORNL-3498
Progress

Contract No. W-7405-eng-26

BIOLOGY DIVISION
SEMIANNUAL PROGRESS REPORT
For Period Ending August 15, 1963

Alexander Hollaender, Director
Stanley F. Carson, Assistant Director

DATE ISSUED

NOV 5 1963

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OAK RIDGE NATIONAL LABORATORY
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Publications and Lectures

PUBLICATIONS

A total of 334 papers and abstracts and 3 book reviews by members of the Biology Division have been published during this report period or are in press. This figure compares with 350 for the same period in 1962 and with 334 for the February 1963 report period. The 83 published papers and 3 book reviews are listed below. The 47 published abstracts and the 201 papers and abstracts in press are not listed.

The proceedings of the 1963 Gatlinburg Symposium, *Macromolecular Aspects of the Cell Cycle*, will be published by the Wistar Institute of Anatomy and Biology as an October supplement to the *Journal of Cellular and Comparative Physiology*.

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Adler, H. I.	Catalase, hydrogen peroxide, and ionizing radiation	*Radiation Res., Suppl.* **3**, 110–29 (1963)
Anderson, N. G. (prepared by)	The Joint National Institutes of Health–Atomic Energy Commission Zonal Centrifuge Development Program: Semiannual Report for period July 1–December 31, 1962	ORNL-3415
Anderson, N. G.	On the origin of life	pp 1–16 in *Lectures in Biological Sciences* (ed. by J. I. Townsend), The University of Tennessee Press, Knoxville, 1963
	Particle separation method	U.S. Patent No. 3,075,694
Asano, Masahide, T. T. Odell, Jr., T. P. McDonald, and A. C. Upton	Radiomimetic agents and x rays in mice and AET protectiveness: *Comparative pathological effects*	*A.M.A. Arch. Pathol.* **75**, 250–63 (1963)
Barber, A. A., Carol Dempster, and N. G. Anderson	A gel filtration method for studies on protein iron-binding	*Clin. Chim. Acta* **8**, 143–45 (1963)
Barnett, W. E., and F. J. de Serres	Fixed genetic instability in Neurospora crassa	*Genetics* **48**, 717–23 (1963)
Bender, M. A, and P. Carolyn Gooch	Persistent chromosome aberrations in irradiated human subjects. II. Three and one-half year investigation	*Radiation Res.* **18**, 389–96 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Bollum, F. J.	"Primer" in DNA polymerase reactions	pp 1–26 in *Progress in Nucleic Acid Research*, vol I (ed. by J. N. Davidson and W. E. Cohn), Academic Press, New York and London, 1963
Bollum, F. J., and R. B. Setlow	Ultraviolet inactivation of deoxyribonucleic acid primer activity. I. Effects of different wavelengths and doses	*Biochim. Biophys. Acta* **68**, 599–607 (1963)
Boyce, Richard, and R. B. Setlow	The action spectra for ultraviolet-light inactivation of systems containing 5-bromouracil-substituted deoxyribonucleic acid: I. *Escherichia coli* 15 $T^-A^-U^-$	*Biochim. Biophys. Acta* **68**, 446–54 (1963)
Brockman, H. E., and F. J. de Serres	Induction of ad-3 mutants of Neurospora crassa by 2-aminopurine	*Genetics* **48**, 597–604 (1963)
Cameron, I. L., and D. M. Prescott	RNA and protein metabolism in the maturation of the nucleated chicken erythrocyte	*Exptl. Cell Res.* **30**, 609–12 (1963)
Celada, Franco, and W. J. Welshons	An immunogenetic analysis of the male antigen in mice utilizing animals with an exceptional chromosome constitution	*Genetics* **48**, 139–51 (1963)
Chu, E. H. Y.	Dose relations in the induction of human chromosome aberrations	pp 217–36 in *Radiation-Induced Chromosome Aberrations* (ed. by Sheldon Wolff), Columbia University Press, New York, 1963
	Mammalian chromosome cytology	*Am. Zool.* **3**, 3–14 (1963)
Cohn, W. E (coeditor)	Progress in Nucleic Acid Research, vol 1 (424 pp)	Edited by J. N. Davidson and W. E Cohn. Academic Press, New York and London, 1963
Congdon, C. C, and A. L. Kretchmar	Increased liver weight in bone marrow chimeras	*Exptl. Mol. Pathol.* **2**, 277–90 (1963)
Conklin, J. W., A. C. Upton, K. W. Christenberry, and T. P. McDonald	Comparative late somatic effects of some radiomimetic agents and x rays	*Radiation Res.* **19**, 156–58 (1963)
Davis, M. L., G. E. Cosgrove, and D. G. Gosslee	X radiation doses and mortality in mouse strains used in transplantation experiments in the Biology Division	ORNL CF-63-2-17
Deering, R. A., and R. B. Setlow	Effects of ultraviolet light on thymidine dinucleotide and polynucleotide	*Biochim. Biophys. Acta* **68**, 526–34 (1963)
Dent, J. N., and J. S. Kirby-Smith	Metamorphic physiology and morphology of the cave salamander, *Gyrinophilus palleucus*	*Copeia* **1**, 119–30 (1963)
de Serres, F. J.	Studies with purple adenine mutants in Neurospora crassa. V. Evidence for allelic complementation among ad-3B mutants	*Genetics* **48**, 351–60 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Doherty, D. G., and Raymond Shapira	Synthesis of D- and L-2-aminobutylisothiourea dihydrobromide isomers and their conversion to guanidothiols, disulfides, and thiazolines	*J. Org. Chem.* **28**, 1339–42 (1963)
Dolin, M. I., and K. B. Jacobson	Carbonyl addition reactions of nicotinamide adenine dinucleotide in frozen alkaline solution	*Biochem. Biophys. Res. Commun.* **11**, 102–6 (1963)
Doria, Gino	Identification of the immune system responsible for the specificity of actively acquired tolerance in mice	*Proc. Natl. Acad. Sci. U.S.* **49**, 281–86 (1963)
Fisher, W. D., and G. B. Cline	A density gradient for the isolation of metabolically active thymus nuclei	*Biochim. Biophys. Acta* **68**, 640–42 (1963)
Fritz, P. J., and K. B. Jacobson	Lactic dehydrogenases: Subfractionation of isozymes	*Science* **140**, 64–65 (1963)
Goff, R. A.	The relation of developmental status of limb formation to x-radiation sensitivity in chick embryos. I. Gross Study	*J. Exptl. Zool.* **151**, 177–200 (1962)
Grell, E. H.	The dose effect of *ma-1*$^+$ and *ry*$^+$ on xanthine dehydrogenase activity in *Drosophila melanogaster*	*Z. Vererbungslehre* **93**, 371–77 (1962)
Hodgson, G. S.	Radiosensitivity of marrow cells responsible for re-establishing erythropoiesis in lethally irradiated mice	*Acta Physiol. Latinoam.* **12**, 365–69 (1962)
Hollaender, Alexander	Acceptance speech at University of Leeds (on occasion of conferment of honorary degrees, Doctor of Science *honoris causa* to Louis Harold Gray, Alexander Hollaender, Alexander Mikhajlovich Kuzin, and Raymond Latarjet)	pp vii–x in *Radiation Effects in Physics, Chemistry, and Biology, Proceedings of the Second International Congress of Radiation Research, Harrogate, England, August 5–10, 1962* (ed. by Michael Ebert and Alma Howard), North-Holland Publishing Company, Amsterdam, 1963
Hollaender, Alexander, and S. F. Carson	Biology Division Semiannual Progress Report for Period Ending February 15, 1963	ORNL-3427
Jacobson, K. B., and Susumu Nishimura	Paper chromatography of transfer ribonucleic acid on diethylaminoethyl-cellulose	*Biochim. Biophys. Acta* **68**, 490–93 (1963)
Jacobson, K. B., and Kazuo Nishio	Studies on plasma lactic dehydrogenase in mice with myeloid leukemia. II. On the site of production of the enzyme	*Cancer Res.* **23**, 344–48 (1963)
Kaji, Akira, Hideko Kaji, and G. D. Novelli	A soluble amino acid incorporating system	*Biochem. Biophys. Res. Commun.* **10**, 406–9 (1963)
Karasaki, Shuichi	Studies on amphibian yolk. 1. The ultrastructure of the yolk platelet	*J. Cell Biol.* **18**, 135–51 (1963)
Kenney, F. T.	Immunochemical analysis of the induction of tyrosine-α-ketoglutarate transaminase in rat liver	*Ann. N.Y. Acad. Sci.* **103**, 1083–92 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Khym, J. X.	The reaction of methylamine with periodate-oxidized adenosine 5′-phosphate	*Biochemistry* **2**, 344–50 (1963)
	The use of amines in liquid-liquid extractions of nucleic acids and related compounds	*Biochemistry* **2**, 401–6 (1963)
Kimball, R. F.	X-ray dose rate and dose fractionation studies on mutation in Paramecium	*Genetics* **48**, 581–95 (1963)
King, Vera M., and G. E. Cosgrove	Intestinal helminths in various strains of laboratory mice	*Lab. Animal Care* **13**, 46–48 (1963)
Kirby-Smith, J. S.	Effects of combined UV and X radiations on chromosome breakage in *Tradescantia* pollen	pp 203–14 in *Radiation-Induced Chromosome Aberrations* (ed. by Sheldon Wolff), Columbia University Press, New York, 1963
Krieg, D. R.	Ethyl methanesulfonate-induced reversion of bacteriophage T4*r*II mutants	*Genetics* **48**, 561–80 (1963)
Lindsley, D. L.	General survey of genetic effects of chromosomal aberrations	pp 273–89 in *Radiation-Induced Chromosome Aberrations* (ed. by Sheldon Wolff), Columbia University Press, New York, 1963
Löbbecke, E. A.	Nitrogen mustard induced mutation in extracellular bacteriophage	*Genetics* **48**, 691–96 (1963)
Maisin, J. R., and D. G. Doherty	Comparative chemical protection to the intestinal and hematopoietic systems of whole-body X-irradiated mice	*Radiation Res.* **19**, 474–84 (1963)
Marin, Guglielmo	*Radiobiology* (ed. by P. L. T. Ilbery), Butterworths, London, 1961, 314 pp, illustrations	*Blood* **21**, 113 (1963) (book review)
Mattingly, Sister M. Augustine	Nuclear protein synthesis in *Vicia faba*	*Exptl. Cell Res.* **29**, 314–26 (1963)
Mazur, Peter	Mechanisms of injury in frozen and frozen-dried cells	pp 59–70 in *Culture Collections: Perspectives and Problems – Proceedings of the Specialists' Conference on Culture Collections, Ottawa, August 1962* (ed. by S. M. Martin), University of Toronto Press, 1963
	Studies on rapidly frozen suspensions of yeast cells by differential thermal analysis and conductometry	*Biophys. J.* **3**, 323–53 (1963)
Miller, O. L., Jr., and G. E. Stone	Fine structure of the oral area of *Tetrahymena patula*	*J. Protozool.* **10**, 280–88 (1963)
Nishimura, Susumu, and G. D. Novelli	Resistance of S-RNA to ribonucleases in the presence of magnesium ion	*Biochem. Biophys. Res. Commun.* **11**, 161–65 (1963)
Nishio, Kazuo, K. B. Jacobson, V. K. Jenkins, and A. C. Upton	Studies on plasma lactic dehydrogenase in mice with myeloid leukemia. I. Relation of enzyme level to course of disease	*Cancer Res.* **23**, 340–43 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Novelli, G. D., and J. M. Eisenstadt	Specific template RNA for β-galactosidase	pp 301–15 in *Informational Macromolecules* (a Symposium held at the Institute of Microbiology of Rutgers, The State University, with support from the National Science Foundation) (ed. by Henry J. Vogel, Vernon Bryson, and J. Oliver Lampen), Academic Press, New York and London, 1963
Oakberg, E. F.	The influence of germ cell stage on reproductive and genetic effects of radiation in mammals	*Monograph Supplement, Diseases of the Nervous System* **24**, 1–6 (1963)
Perkins, E. H., and Martha R. Leonard	Specificity of phagocytosis as it may relate to antibody formation	*J. Immunol.* **90**, 228–37 (1963)
Popp, R. A.	Hemoglobin loci: Mice classified for their *Hb* and *Sol* alleles	*Science* **140**, 893–94 (1963)
	Mammalian hemoglobins	pp 299–322 in *Methodology of Mammalian Genetics* (ed. by W. J. Burdette), Holden-Day Inc., San Francisco, 1963
Prescott, D. M.	Discussion for Part II	pp 143–48 in *Informational Macromolecules* (a Symposium held at the Institute of Microbiology of Rutgers, The State University, with support from the National Science Foundation) (ed. by Henry J. Vogel, Vernon Bryson, and J. Oliver Lampen), Academic Press, New York and London, 1963
	Division of Labor in Cells, by G. H. Bourne, Academic Press, New York, 1962, 248 pp	*Am. Scientist* **51**, 205A–206A (1963) (book review)
	RNA and protein replacement in the nucleus during growth and division and the conservation of components in the chromosome	pp 111–28 in *Cell Growth and Cell Division, Symposia of the International Society for Cell Biology* (ed. by R. J. C. Harris), vol 2, Academic Press, New York, 1963
	Tritiated precursors employed for the study of nuclear function and nuclear replication	pp 71–84 in *Use of Radioisotopes in Animal Biology and the Medical Sciences, Proceedings of a Conference held in Mexico City, November 21–December 1, 1961*, vol 1, Academic Press, New York, 1962
Prescott, D. M., and M. A Bender	Autoradiographic study of chromatid distribution of labeled DNA in two types of mammalian cells *in vitro*	*Exptl. Cell Res.* **29**, 430–42 (1963)
Randolph, M. L.	First-collision doses produced by fast neutron fluxes of wide energy distributions	*Radiation Res.* **19**, 492–511 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Russell, Liane B.	Mammalian x-chromosome action: Inactivation limited in spread and in region of origin	*Science* **140**, 976–78 (1963)
Russell, W. L., and E. F. Oakberg	The cellular basis and aetiology of the late effects of irradiation of fertility in female mice	pp 229–32 in *Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation* (ed. by R. J. C. Harris), Academic Press, London and New York, 1963
Setlow, R. B., and Richard Boyce	The action spectra for ultraviolet-light inactivation of systems containing 5-bromouracil-substituted deoxyribonucleic acid. II. Bacteriophage T4	*Biochim. Biophys. Acta* **68**, 455–61 (1963)
Setlow, R. B., and W. L. Carrier	Identification of ultraviolet-induced thymine dimers in DNA by absorbance measurements	*Photochem. Photobiol.* **2**, 49–57 (1963)
Simpson, L. P., J. S. Kirby-Smith, and M. L. Randolph	Electron spin resonance phenomena in photodynamic dyes (Letter to the editor)	*Nature* **199**, 243–45 (1963)
Smith, L. H., and Otto Vos	Radiation sensitivity of mouse lymph node cells relative to their proliferative capacity *in vivo*	*Radiation Res.* **19**, 485–91 (1963)
Snider, P. J.	Genetic evidence for nuclear migration in basidiomycetes	*Genetics* **48**, 47–55 (1963)
Vu Thi Suu, C. C Congdon, and A. L. Kretchmar	Increase in lysozyme activity in kidneys of irradiation chimeras	*Proc. Soc. Exptl. Biol. Med.* **113**, 481–85 (1963)
Upton, A. C.	Leukemogenesis – role of viruses and cytological aspects	pp 67–82 in *Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation* (ed. by R. J. C. Harris), Academic Press, London and New York, 1963
Upton, A. C., J. W. Conklin, T. P. McDonald, and K. W. Christenberry	Preliminary studies on late somatic effects of radiomimetic chemicals	pp 171–75 in *Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation* (ed. by R. J. C. Harris), Academic Press, London and New York, 1963
Upton, A. C., M. A. Kastenbaum, and J. W. Conklin	Age-specific death rates of mice exposed to ionizing radiation and radiomimetic agents	pp 285–97 in *Cellular Basis and Aetiology of Late Somatic Effects of Ionizing Radiation* (ed. by R. J. C. Harris), Academic Press, London and New York, 1963
Urso, Paul, and Takashi Makinodan	The roles of cellular division and maturation in the formation of precipitating antibody	*J. Immunol.* **90**, 897–907 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Volkin, Elliot, and Anna Ruffilli	The dissociation of T2-specific RNA, protein, and DNA syntheses	pp 55–66 in *Informational Macromolecules* (a Symposium held at the Institute of Microbiology of Rutgers, The State University, with support from the National Science Foundation) (ed. by Henry J. Vogel, Vernon Bryson, and J. Oliver Lampen), Academic Press, New York and London, 1963
Volkin, Elliot, and Drew Schwartz	Nucleic acid metabolism of germinating corn seedlings	*Bull. Res. Council Israel* (S. Hestrin Memorial Issue) **11**, 387–88 (1963)
von Borstel, R. C.	*New Patterns in Genetics and Development*, by C. H. Waddington, Columbia University Press, New York, 1962, 271 pp	*Am. Scientist* **51**, 174A and 178A (1963) (book review)
	Preface and Postcript to Refresher Course in Recent Advances in Cytogenetics and Developmental Genetics	*Am. Zool.* **3**, 2 and 87–95 (1963)
von Borstel, R. C., E. G. Calef, and A. L. Colomb	Exposure of biological specimens to high fluxes of thermal neutrons	ORNL TM-489
Wallace, R. A., and Shuichi Karasaki	Studies on amphibian yolk. 2. The isolation of yolk platelets from the eggs of *Rana pipiens*	*J. Cell Biol.* **18**, 153–66 (1963)
Welshons, W. J.	Cytological contributions to mammalian genetics	*Am. Zool.* **3**, 15–22 (1963)
Williams, Anna M., and F. J. Bollum	Inhibition of enzymatic DNA synthesis by certain pathological human sera	*Proc. Soc. Exptl. Biol. Med.* **112**, 701–6 (1963)
Wolff, Sheldon	Extended comments to paper by H. J. Evans: Chromosome aberrations and target theory	pp 8–40 in *Radiation-Induced Chromosome Aberrations* (ed. by Sheldon Wolff), Columbia University Press, New York, 1963
Wolff, Sheldon (editor)	Radiation-Induced Chromosome Aberrations (304 pp)	Edited by Sheldon Wolff, Columbia University Press, New York and London, 1963
Woodward, J. M.	*Pseudomonas aeruginosa* infection and its control in the radiobiological research program at Oak Ridge National Laboratory	*Lab. Animal Care* **13**, 20–24 (1963)
Wust, C. J.	Interference with antibody neutralization by coenzyme and reducing agents	*Ann. N.Y. Acad. Sci.* **103**, 849–57 (1963)
Wust, C. J., and G. D. Novelli	Ribonucleoprotein-bound antibody to the enzyme triose phosphate dehydrogenase	*J. Immunol.* **90**, 734–40 (1963)
Yamada, Tuneo, and Shuichi Karasaki	Nuclear RNA synthesis in newt iris cells engaged in regenerative transformation into lens cells	pp 595–604 in *Developmental Biology* (ed. by J. Brachet, E. Hadorn, and P. Weiss), vol 7, Academic Press, New York, 1963

LECTURES

During the period February to August 1963, members of the Biology Division presented 182 lectures and speeches compared with 252 for the corresponding period of 1962. Of these, 82 were before professional societies at home and abroad, 10 were given as part of the Traveling Lecture Program, and the remainder was presented on invitation of universities, laboratories, and scientific organizations.

Details on speaking activities outside the continental United States will be found in the section "Foreign Travel." Educational activities are described under "Education."

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I.	Environments that permit life	13th Session, Oak Ridge Science Lecture Demonstration Program, ORINS
	Genetic analysis in *Escherichia coli* K-12 of sensitivity to ionizing radiation [*Bacteriol. Proc.*, p 29 (1963)]	Am. Soc. Microbiol. (63rd Annual Meeting), Cleveland, Ohio
Albright, J. F.	Some dynamic aspects of antibody production	Embryology Training Program (sponsored by Carnegie Institution of Washington), Woods Hole, Mass.
Albright, J. F. (Takashi Makinodan and Rachel R. Carter)	Relationship between antigen dose and antibody production in secondary hemaglutinin response [*Federation Proc.* **22**, 266 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Assoc. Immunol.), Atlantic City, N.J.
Anderson, N. G.	Alterations in the molecular scans of cells and tissues produced by drugs	Gordon Research Conference on Toxicology and Safety Evaluations, Meriden, N.H.
	Development of zonal centrifuges [*Federation Proc.* **22**, 674 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Physiol. Soc.), Atlantic City, N.J.
	In search of viruses	Sigma Xi, University of South Carolina, Columbia
	New methods of virus isolation	13th Session, Oak Ridge Science Lecture Demonstration Program, ORINS
	Topics in molecular physiology	University of South Carolina, Columbia
Arnold, W. A.	Chloroplasts as semiconductors	Am. Physical Soc., Washington, D.C.
	Photosynthesis	Solar Energy Symposium, University of Florida, Gainesville
Baum, R. H. (and M. I. Dolin)	Presumptive evidence for the presence of a quinone in *Streptococcus faecalis*, 10 C1 [*Bacteriol. Proc.*, p 96 (1963)]	Am. Soc. Microbiol. (63rd Annual Meeting), Cleveland, Ohio
Becker, R. R. (and Fumio Sawada)	Enzymatic properties of polypeptidyl ribonucleases [*Federation Proc.* **22**, 419 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Bell, A. G.	Irradiation-induced polyploidy in human leukocyte cultures	Am. Soc. Human Genetics, New York

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Bender, M. A	Induced aberrations in human chromosomes [*Am. J. Pathol.* **43**, 26A (1963)]	Am. Assoc. Pathol. and Bacteriol., Symposium on Human Chromosome Abnormalities, Cincinnati, Ohio
	Space biology	Oak Ridge Section Am. Inst. Chem. Eng., Oak Ridge, Tenn.
Bertsch, W. F.	Solid state phenomena in photosynthetic energy conversion	1. Michigan State University, East Lansing 2. University of Maryland, College Park
Bollum, F. J.	Intermediate states in enzymatic DNA synthesis	Symposium on Macromolecular Aspects of the Cell Cycle, Gatlinburg, Tenn. (ORNL Biology Division Symposium)
	Enzymatic Synthesis of DNA	Mellon Institute, Pittsburgh, Pa.
	Nature of calf thymus DNA polymerase products	Gordon Research Conference on Nucleic Acids, New Hampton, N.H.
	Nature of DNA-polymerase products	University of Michigan, Ann Arbor
	Some new and old ideas about DNA	Montefiore Hospital Institute, Pittsburgh, Pa.
	Studies on calf thymus DNA polymerase products	Cold Spring Harbor Symp. Quant. Biol., Cold Spring Harbor, L.I., N.Y.
Bollum, F. J. (and G. E. Houts)	Incorporation of H^3-DNA primer into calf thymus DNA polymerase product [*Federation Proc.* **22**, 462 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Burger, C. L.	Zonal centrifugation	Gordon Research Conference on Separation and Purification, New London, N.H.
Cameron, I. L.	The constant DNA synthetic period in somatic cells of homeotherms	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Cammarano, Piero (and G. D. Novelli)	Studies on the mouse liver amino acid incorporating system [*Federation Proc.* **22**, 581 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Carson, S. F.	1. Relation between growth of bacteria and ATP generation 2. Theoretical energy requirements for growth of bacteria	University of Georgia, Athens
Carson, S. F. (E. F. Phares, Mary V. Long, and S. F. Carson)	Negative ion mass analysis of succinic acid in studies on the mechanism of the methylmalonyl isomerase reaction [*Bacteriol. Proc.*, p 109 (1963)]	Am. Soc. Microbiol. (63rd Annual Meeting), Cleveland, Ohio
Cattanach, B. M.	Position effects in the mouse in relation to the inactive-X hypothesis	Columbia University, New York

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Chu, E. H. Y.	Abnormalities of human sex chromosomes	Goucher College, Baltimore, Md.
	Chromosome cytology and evolution in primates	Morgan State College, Baltimore, Md.
	The molecular basis for chromosome aberrations induced by ultraviolet radiation	M. D. Anderson Hospital and Tumor Institute, University of Texas, Houston
	Ultraviolet-induced mammalian chromosome aberrations	1. Institute for Cancer Research, Philadelphia, Pa. 2. Western Reserve University, Cleveland, Ohio
Cleffman, Günter	Cell physiological implications in the development of the agouti pattern	1. Institute for Cancer Research, Philadelphia, Pa. 2. Harvard Medical School, Boston, Mass. 3. Brown University, Providence, R.I.
	The development of the agouti pattern in mice	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Cohn, W. E	Determination of nucleotide structures	Centre Européen de Recherches Nucléaire, Geneva, Switzerland
	New nucleotides	Société de Chimie Biologique, Paris, France
	New nucleotides, structure determination of nucleotides, and chromatography	Institut de Biologie Physico-Chimique, Paris, France
	Pseudouridine	1. Farbwerken Hoechst, Frankfurt, Germany 2. University of Marburg, Marburg, Germany 3. University of Frankfurt, Frankfurt, Germany 4. CIBA Research Dept., CIBA Ltd., Basel, Switzerland 5. University of Glasgow, Glasgow, Scotland 6. Bradford Institute of Technology, Bradford, England 7. Laboratorio Internazionale di Genetica e Biofisica, Naples, Italy 8. Stazione Zoologica, Naples, Italy
Congdon, C. C	Early histologic effects of antigenic stimulation in the mouse spleen [*Federation Proc.* **22**, 374 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Exptl. Pathol.), Atlantic City, N.J.
	Protection and recovery in mammals	Oak Ridge School of Reactor Technology (ORSORT), Oak Ridge, Tenn.
	Summary of meeting	International Symposium on the Control of Cell Division and Induction of Cancer, Lima, Peru, and Cali, Colombia

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Congdon, C. C (A. L. Kretchmar and C. C Congdon)	Serine metabolism in mice carrying homologous bone marrow grafts [*Federation Proc.* **22**, 392 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Assoc. Immunol.), Atlantic City, N.J.
Cosgrove, G. E.	Acute effects of radiation on mammals	Oak Ridge School of Reactor Technology (ORSORT), Oak Ridge, Tenn.
	Mammalian radiation biology	NSF-AEC Summer Institutes in Radiation Biology for Science Teachers
	1. Four lectures	1. Florida State University, Tallahassee
	2. Four lectures and demonstrations	2. Wayne State University, Detroit, Mich.
Cosgrove, G. E. (A. C. Upton, C. C Congdon, D. G. Doherty, and D. G. Gosslee)	Effects of AET and bone marrow on delayed somatic effects of radiation in mice [*Radiation Res.* **19**, 231 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
Darden, E. B.	Effects of radiation on biological organisms	US Army Nuclear Science Seminar, ORINS, Oak Ridge, Tenn.
Darden, E. B. (Margaret Bradley and A. C. Upton)	Influence of early sublethal whole-body irradiation and of breeding status on thermal aging of collagen fibers in the mouse [*Radiation Res.* **19**, 190–91 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
de Serres, F. J.	1. Chemical mutagenesis in *Neurospora* 2. The effect of environment on allelic complementation 3. The effect of genetic constitution on recombination	Goucher College, Baltimore, Md.
Doherty, D. G.	Enzymes, the biological catalysts	Spring Hill College, Spring Hill, Ala.
	Protective effect of mixtures of glutathione and mercaptoethyl guanidine or mercaptoethylamine against X radiation [*Federation Proc.* **22**, 235 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Dolin, M. I.	Carbonyl addition reactions of nicotinamide adenine dinucleotide in frozen alkaline solution	1. Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 2. University of Illinois, Urbana
Dolin, M. I. (and K. B. Jacobson)	A weak alkali modification product of nicotinamide adenine dinucleotide [*Federation Proc.* **22**, 471 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Fritz, P. J.	Resolution of LDH isozymes into multiple fractions [*Federation Proc.* **22**, 241 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Giudice, Giovanni	Restitution of whole larvae from disaggregated cells of sea urchin embryos	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Goodman, Joan W.	Origin and transplantation potentialities of peritoneal fluid cells	University of Chile, Santiago, Chile
	Stem cells of the hemopoietic and lymphatic tissues	International Symposium on the Control of Cell Division and Induction of Cancer, Lima, Peru, and Cali, Colombia

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Greenman, D. L. (and F. T. Kenney)	A cell-free amino acid incorporating system from uterine tissue of the rat [*Federation Proc.* **22**, 581 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Gude, W. D.	Basic radiobiology	US Army Nuclear Science Seminar, ORINS, Oak Ridge, Tenn.
	Biological effects of radiation on mammals	Tenn. Acad. Sci. – Visiting Scientist Program (NSF-TSDE), Alcoa High School, Alcoa, Tenn.
	Research in the Biology Division and its organization	Tenn. Acad. Sci. – Visiting Scientist Program (NSF-TSDE), Douglass High School, Kingsport, Tenn.
Ginsberg, D. M. (and John Jagger)	Photoreactivation and dark recovery of ultraviolet killing in *Escherichia coli* strain 15 TAU [*Radiation Res.* **19**, 233 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
Haber, A. H.	Inadequacy of the cell theory for interpreting morphogenesis	University of Texas, Austin
	Roles of cell division in plant growth and differentiation	Florida State University, Tallahassee
Haber, A. H. (and D. E. Foard)	Interpretations concerning cell division and growth	Fifth International Conference on Natural Plant Growth Regulators, Gif-sur-Yvette, France
Hollaender, Alexander	Biology at ORNL	Student Trainee Program, ORNL, Oak Ridge, Tenn.
	1. Opening and closing remarks at Lima, Peru 2. Similar remarks at Cali, Colombia 3. Lecture and discussion leader at Lima on International Cooperation in Biological Research, especially as applicable to Latin America	International Symposium on Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia
	Possibilities for cooperation between San Marcos University and the Biology Division, Oak Ridge National Laboratory, in Biology	San Marcos University, Lima, Peru
Jacobson, K. B.	Paper chromatography of transfer RNA [*Federation Proc.* **22**, 643 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Jagger, John	Ultraviolet radiation and the origin of life	13th Session, Oak Ridge Science Lecture Demonstration Program, ORINS, Oak Ridge, Tenn.
Jagger, John (R. S. Stafford and R. J. Mackin, Jr.)	Photoreactivation of killing of *Streptomyces griseus* conidia by far ultraviolet and vacuum ultraviolet radiation (1500-2600 A) [*Radiation Res.* **19**, 233–34 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
James, T. W.	Another plan for cell fission	Florida State University, Tallahassee

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Jenkins, V. K. (A. C. Upton and N. G. Anderson)	Transmission of radiogenic myeloid leukemia in the RF mouse [*Proc. Am. Assoc. Cancer Res.* **4**, 31 (1963)]	Am. Assoc. Cancer Res. (54th Annual Meeting), Toronto, Canada
Kaji, Akira (and Hideko Kaji)	A soluble amino acid incorporation system isolated from *E. coli* [*Federation Proc.* **22**, 302 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Karasaki, Shuichi	Amphibian yolk platelets, their ultrastructure and role in development	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Khym, J. X.	The use of amines in liquid-liquid extractions of nucleic acid derivatives [*Abstr. of papers, 144th Meet., Am. Chem. Soc.* (1963), p 4C]	Am. Chem. Soc., Los Angeles, Calif.
Kimball, R. F.	Cellular aspects of radiation damage	Oak Ridge School of Reactor Technology (ORSORT), Oak Ridge, Tenn.
	Intraclonal genetics of Paramecium and Tetrahymena	Longwood College, Farmville, Va.
	The postirradiation period in mutation induction	Summer Symposium on Radiation Biology, Ann Arbor, Mich.
	Studies on reparable premutational X-ray damage [*Radiation Res.* **19**, 183–84 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
Kretchmar, A. L. (and C. C Congdon)	RNA and DNA in liver of irradiation chimeras	Bone Marrow Transplantation and Irradiation Protection Meeting, Atlantic City, N.J.
	Serine metabolism in mice carrying homologous bone marrow grafts	Federation Am. Soc. Exptl. Biol. (Am. Assoc. Immunol.), Atlantic City, N.J.
Lanigan, Sister M. Regina (and H. D. Peck, Jr.)	The mechanism of sulfate reduction in extracts of *Desulfovibrio orientis* [*Bacteriol. Proc.*, p 123 (1963)]	Am. Soc. Microbiol. (63rd Annual Meeting), Cleveland, Ohio
Mans, R. J.	The evolution of an amino acid incorporating system from maize	University of Georgia, Athens
McArthur, W. H. (A. L. Kretchmar and C. C Congdon)	Study of nitrogen balance in irradiated mice given foreign bone marrow	Bone Marrow Transplantation and Irradiation Protection Meeting, Atlantic City, N.J.
Nishimura, Susumu	Effect of *Bacillus subtilis* and pancreatic ribonuclease on S-RNA and amino acid incorporation [*Federation Proc.* **22**, 643 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Novelli, G. D.	Control mechanisms in the cell-free synthesis of protein	Argonne National Laboratory, Argonne, Ill.
	Control mechanisms in protein synthesis	Francis Delafield Hospital, Columbia University, New York
	Horizons in chemistry	Marquette University, Milwaukee, Wis.
	How genes control enzymes	NSF Summer Science Program for High School Students, Virginia Polytechnic Institute, Blacksburg, Va.
	Protein synthesis (series of 4 lectures)	Tulane University, New Orleans, La.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Oakberg, E. F.	The effects of radiation upon germ cell populations	Symposium on Supervoltage Irradiation and Genetics, City of Hope Medical Center, Duarte, Calif.
	Effects of radiation on mammalian germ cells	Oak Ridge School of Reactor Technology (ORSORT), Oak Ridge, Tenn.
	Genetic effects of radiation	US Army Nuclear Science Seminar, ORINS, Oak Ridge, Tenn.
	Radiation response of the mammalian oocyte	USAEC, Division of Biology and Medicine, Round-Table Discussion on the Role of Large Animal Studies in Elucidating the Gonadal Effects of Irradiation in Humans, Germantown, Pa.
Odell, T. T., Jr.	Biological effects of radiation on mammals	US Army Nuclear Science Seminar, ORINS, Oak Ridge, Tenn.
Peck, H. D., Jr.	1. Energy yielding systems of certain autotrophic bacteria 2. Properties of APS-reductase from *D. desulfuricans*	Tulane University, New Orleans, La.
	The role of sulfur-containing nucleotides in the metabolism of inorganic sulfur compounds	University of Kansas, Lawrence
Peck, H. D., Jr. (and T. E. Deacon)	Properties of adenosine 5′-phosphosulfate reductase from *Desulfovibrio desulfuricans* [*Federation Proc.* **22**, 356 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Perkins, E. H.	Changes in pool size of potentially competent Ab-forming cells from spleens of nonprimed and primed donors grown in *in vivo* cultures [*Federation Proc.* **22**, 380 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Soc. Exptl. Pathol.), Atlantic City, N.J.
Popp, R. A.	The effects of radiation on mammals	13th Session, Oak Ridge Science Lecture Demonstration Program, ORINS, Oak Ridge, Tenn.
Prescott, D. M.	The cell life cycle	University of Chicago, Chicago, Ill.
	Chromosome replication	University of Colorado Medical School, Denver
	Function of the cell nucleus	Northwestern State College, Alva, Okla.
	The growth of the cell	Phillips University, Enid, Okla.
	Histone turnover and related matters	World Histone Biology Conference, San Diego, Calif.
	RNA and protein metabolism and intracellular migration during the cell life cycle	International Symposium on the Control of Cell Division and Induction of Cancer, Lima, Peru, and Cali, Colombia
	Tissue culture (2 lectures and laboratories)	Tissue Culture Course, University of Wisconsin, Madison
	The turnover of nuclear proteins in Amoeba	National Academy of Sciences (100th Annual Meeting), Washington, D.C.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Prescott, D. M. (and M. A Bender)	Synthesis and behavior of nuclear proteins during the cell life cycle	Symposium on Macromolecular Aspects of the Cell Cycle (ORNL Biology Division Symposium), Gatlinburg, Tenn.
Prescott, D. M. (and Romance F. Carrier)	Proteins in nuclear-cytoplasmic interaction in *Amoeba proteus*	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Randolph, M. L.	Electron spin resonance studies of irradiated biochemical and biological systems	Union Carbide Magnetic Resonance Meeting, Cleveland, Ohio
	Free radical studies on irradiated biochemicals	Medical College of Virginia, Richmond
Russell, Liane B.	Genetic effects of radiation on mammals	Oak Ridge School of Reactor Technology (ORSORT), Oak Ridge, Tenn.
	Mechanisms for sex chromosome errors in experimental mammals	Symposium on Mammalian Sex Chromosomes, The Endocrine Society, Atlantic City, N.J.
Russell, W. L.	Genetic effects of radiation	University of California, Berkeley
	The genetic effects of radiation in mammals	Symposium on Supervoltage Irradiation and Genetics, City of Hope Medical Center, Duarte, Calif.
	Recent experiments on the genetic effects of radiation	Am. Industrial Hygiene Assoc. (24th Annual Meeting), Cincinnati, Ohio
Sado, Toshihiko (and Martha R. Leonard)	Autoradiographic studies on the proliferative capacity of antibody-producing cells [*Radiation Res.* **19**, 227–28 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
Schuel, Herbert (N. G. Anderson and S. R. Tipton)	Tissue fractionation in zonal centrifuges	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Schuel, Herbert (and Regina Schuel)	Aggregation reactions and acid production in rat liver brei induced by divalent cations [*Federation Proc.* **22**, 178 (1963)]	Federation Am. Soc. Exptl. Biol. (Am. Physiol. Soc.), Atlantic City, N.J.
Setlow, Jane K.	Photoreactivation and molecular changes produced by UV	Symposium on Molecular Changes Responsible for the Effects of UV on Nucleic Acids, Radiation Research Society, Milwaukee, Wis.
Setlow, R. B.	DNA: its structure, function, and inactivation	Physics Division Seminar, ORNL, Oak Ridge, Tenn.
	Macromolecular changes responsible for UV effects on DNA	Bell Telephone Laboratories, Murray Hill, N.J.
	Mechanism of ultraviolet injury in cells	Am. Physical Soc., Washington, D.C.
Seto, Frank	Decrease in embryonic splenomegaly by X-irradiation [*Radiation Res.* **19**, 228 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
Seto, Frank (and J. F. Albright)	Quantitative studies of the splenomegaly phenomenon in the chick embryo	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Smith, L. H.	Fe^{59} incorporation by spleens of lethally irradiated mice injected with isologous bone marrow [*Radiation Res.* **19**, 196–97 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
	Radiation protective and recovery measures	US Army Nuclear Science Seminar, ORINS, Oak Ridge, Tenn.
	Uses of isotopes in determining tissue viability	Conference on Tissue Viability, Roswell Park Memorial Institute, Buffalo, N.Y.
Stallcup, W. B. (Gustavo Cudkowicz and W. L. Hughes)	Measurement of the radiation sensitivity of mouse bone marrow cells by use of I^{131}-labeled iododeoxyuridine (I^{131} UdR) [*Radiation Res.* **19**, 196 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.
Stapleton, G. E.	Protection and recovery in microorganisms	Oak Ridge School of Reactor Technology (ORSORT), Oak Ridge, Tenn.
	Requirements for development of radioresistance in *Escherichia coli* [*Bacteriol. Proc.*, p 50 (1963)]	Am. Soc. Microbiol. (63rd Annual Meeting), Cleveland, Ohio
Takata, Chinami (and J. F. Albright)	Appearance of lens-specific antigens during lens regeneration in the urodele	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Tyndall, R. L. (K. B. Jacobson and Ernestine Teeter)	Uninfected and Coxsackie B3 virus infected HeLa cell RNA [*Bacteriol. Proc.*, p 129 (1963)]	Am. Soc. Microbiol. (63rd Annual Meeting), Cleveland, Ohio
Upton, A. C.	Cancer and aging: Implications of studies with ionizing radiation	Francis Delafield Hospital, Columbia University, New York
	Comparative aspects of carcinogenesis by exposure to ionizing radiation	International Symposium on the Control of Cell Division and Induction of Cancer, Lima, Peru, and Cali, Colombia
	New developments in experimental biology of importance to obstetrics and gynecology	East Tennessee Obstetrical and Gynecological Society, Knoxville
	Relation of studies in man and animals (Discussion)	Symposium on Epidemiological Studies in Human Populations Exposed to Ionizing Radiation, Radiation Research Society, Milwaukee, Wis.
von Borstel, R. C.	Effects of radiation on germ cells of insects: Dominant lethals, gamete inactivation, and gonial cell killing	Symposium on the Use and Application of Radioisotopes and Radiation in the Control of Plant and Animal Insect Pests (sponsored by IAEA and the FAO, UN), Athens, Greece
	Radiation-induced dominant lethality in insects	International Laboratory of Genetics and Biophysics, Naples, Italy
	Spontaneous and induced mutations in yeast	Radium Institute, Paris, France
Walburg, H. E., Jr. (and E. I. Mynatt)	Further studies on the fate of bacteria injected into irradiated mice [*Radiation Res.* **19**, 227 (1963)]	Radiation Research Society (11th Annual Meeting), Milwaukee, Wis.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Wallace, R. A.	Biochemistry of yolk	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.
Wust, C. J.	Biochemical studies on the mechanism of antibody synthesis	1. University of North Carolina, Chapel Hill 2. Research Colloquium of the School of Basic Medical Sciences, University of Tennessee, Memphis 3. Vanderbilt University, Nashville, Tenn.
Yamada, Tuneo	RNA synthesis in tissue transformation	National Cancer Institute, Bethesda, Md.
	1. Competence and the pattern of macromolecular synthesis (panel) 2. Synthesis of macromolecules in Wolffian lens regeneration	Sixth International Embryological Conference, Helsinki, Finland
Yamada, Tuneo (F. J. Finamore and Tuneo Yamada)	Demonstration: Synthesis of acid-soluble and acid-insoluble RNA's during embryonic development	Sixth International Embryological Conference, Helsinki, Finland
Yamada, Tuneo (Chinami Takata and Marion E. Roesel)	The pattern of synthesis of macromolecules in urodelan lens regeneration	Southeastern Developmental Biol. Conf. (7th), Knoxville, Tenn.

VISITING LECTURERS

During the six months preceding this report, 54 lectures were given on the Biology Division Seminar program by guest speakers from scientific institutions and universities in this country and abroad. Visiting lecturers included scientists from Argentina, Brazil, Canada, Germany, Israel, Italy, Japan, Scotland, South Africa, South Vietnam, Switzerland, and Wales.

SPEAKER	AFFILIATION	SUBJECT
B. I. Balinsky	Department of Zoology, University of Witwatersrand, Johannesburg, S. Africa	The origin and differentiation of cytoplasmic organoids during oogenesis in the frog
Issac Berenblum	The Weizmann Institute of Science, Rehovoth, Israel	The role of the two-stage mechanism of carcinogenesis in radiobiological research
S. G. Bradley	Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minn.	Genetics of actinomycetes in comparison and provocation to other bacterial systems
M. K. Brakke	U.S. Department of Agriculture, Agricultural Research Service Plant Pathology Department, University of Nebraska, Lincoln	Virus isolation in density gradients

SPEAKER	AFFILIATION	SUBJECT
J. G. Brewen	Mergenthaler Laboratory for Biology, The Johns Hopkins University, Baltimore, Md.	Dependence of observed chromosome breakage on dose rate
W. R. Bryan	Laboratory of Viral Oncology, National Cancer Institute, Bethesda, Md.	Some biological consideration of the tumor viruses
Harris Busch	Department of Pharmacology, Baylor University College of Medicine, Texas Medical Center, Houston, Tex.	Nucleoli of tumor cells
N. P. Buu-Hoi	Atomic Energy Commission in South Vietnam, South Vietnam	Some recent experiments in chemical carcinogenesis
R. S. Caldecott	Division of Biology and Medicine, U.S. Atomic Energy Commission, Washington, D.C.	Postirradiation environmental effects on seeds
D. L. D. Caspar	Children's Cancer Research Foundation Inc., Boston, Mass.	Design and construction of regular virus particles
Ruggero Ceppellini	Istituto di Genetica Medica, Torino, Italy	Problems of homotransplantation in man
H. Cottier	Medical Research Center, Brookhaven National Laboratory, Upton, L.I., N.Y.	New formation and growth of germinal centers during secondary response to tetanus toxoid in mice: an autoradiographic study
Roy Davies	Biology Department, Brookhaven National Laboratory, Upton, L.I., N.Y.	Some studies on relationship between radiation-induced chromosome aberrations and loss of reproductive integrity
R. S. Edgar	Division of Biology, California Institute of Technology, Pasadena	Studies on temperature-sensitive mutants of bacteriophage T4
R. H. Epstein	Laboratoire de Biophysique, Université de Genève, Geneva, Switzerland	Studies of temperature sensitive and amber mutations and bacteriophage T4
Giulio Fermi	Max-Planck-Institut für Biologie, Tübingen, Germany	Optomotor reactions in the housefly
Robert Fujimura	Department of Biochemistry, University of Wisconsin, Madison	Study on RNA of bacteriophage R17
Franco Graziosi	Laboratorio Internazionale di Genetica e Biofisica, Naples, Italy	Biophysical and biochemical studies of phage alpha
Lawrence Grossman	Graduate Department of Biochemistry, Brandeis University, Waltham, Mass.	The effects of ultraviolet on the structure and function of nucleic acids
E. Grundmann	Pathologisches Institut der Universität Freiburg, Freiburg, Germany	How the cancer cell develops: Investigations of carcinogenesis in rat liver by diethylnitrosamine
Philip Hanawalt	W. W. Hansen Laboratories of Physics, Stanford University, Stanford, Calif.	On the involvement of RNA synthesis in thymineless death
Ruth F. Hill	Department of Radiology, College of Physicians & Surgeons of Columbia University, New York	Genetic aspects of UV sensitivity
Carroll Johnson	Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.	The packing of polypeptide chains

SPEAKER	AFFILIATION	SUBJECT
H. E. Johns (joint seminar with Michael Rauth)	Ontario Cancer Institute, Toronto, Canada	UV effects of TpT and UpU UV action spectra of a variety of RNA and DNA viruses
William Konigsberg	The Rockefeller Institute, New York	The structure of adult human hemoglobin
Rose M. Litman	The University of Colorado Medical School, Denver	Enzymatic synthesis of transforming DNA
T. R. Manney	Donner Laboratory and Donner Pavilion, University of California, Berkeley	Preliminary studies of a super suppressor with the tryptophan synthetase system in yeast
Donald Marvin	Max-Planck-Institut für Virusforschung, Tübingen, Germany	fd bacteriophage: a fibrous phage containing single-stranded DNA
Rue Ishida Masahiro	Kyoto University Research Reactor Institute, Kyoto, Japan	Chloroplast DNA in chlamydomonas
J. M. Mitchison	Department of Zoology, University of Edinburgh, Edinburgh, Scotland	Growth during the cell cycle in a fission yeast
R. J. Neff	Department of Biology, Division of Molecular Biology, Vanderbilt University, Nashville, Tenn.	The mechanism of differentiation in a soil amoeba
Maurice Ogur	Department of Microbiology, Southern Illinois University, Carbondale	A glutamic acid requiring mutant in yeast
M. H. Patrick	University of Chicago, Chicago, Ill.	Repair of primary radiation damage in yeast
Walter Plaut	Department of Zoology, The University of Wisconsin, Madison	Pulse labeling studies on Drosophila salivary gland chromosomes
Wolf Prensky	Biology Department, Brookhaven National Laboratory, Upton, L.I., N.Y.	The mechanism of 5-aminouracil induced synchrony of cell division
Jose Reissig	University of Buenos Aires, Buenos Aires, Argentina	Genetic control of arginine and pyrimidine synthesis in Neurospora
D. R. Ridgeway	Department of Biophysics and Biometry, Medical College of Virginia, Richmond	Polarimetric properties of serum albumin
F. A. Rose	Department of Biochemistry, University College of South Wales and Monmouthshire, Cardiff, Wales	Tyrosine sulphate
Barnett Rosenberg	Biophysics Department, Michigan State University, Lansing	Photoconductive model of the visual receptor process
Giorgio Schreiber	Instituto de Biologia, Universidade de Minas Gerais, Minas Gerais, Brazil	Cytometric and cytochemical researches on reduplication series of nuclear components (DNA, nuclear volume and nucleolus)
Raymond Shapira	Department of Biochemistry, Emory University, Atlanta, Ga.	Radiation induced aggregation of bovine pancreatic ribonuclease
R. M. S. Smellie	Department of Biochemistry, The University, Glasgow, Scotland	The biosynthesis of RNA in mammalian cells infected with an RNA virus

SPEAKER	AFFILIATION	SUBJECT
C. E. Smith	Laboratory of Molecular Biology, National Institute of Arthritis & Metabolic Diseases, Washington, D.C.	The physical chemistry of actinomycin—DNA interaction
T. E. Stubblefield	Max-Planck-Institut für Virusforschung, Tübingen, Germany	Architecture of Mause-Elberfeld virus
Jaciento J. Vazquez	Department of Pathology, Duke University Medical Center, Durham, N.C.	Immunopathology of the antigen-antibody complex reaction
Neil Wald	University of Pittsburgh, Graduate School of Public Health, Pittsburgh, Pa.	A consistent extra marker chromosome in granulocytic leukemia of the RF mouse
P. M. B. Walker	Department of Zoology, University of Edinburgh, Edinburgh, Scotland	Hypochromicity of nucleoprotein in intact sperm heads and nuclei
Roger Weil	Gates & Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena	Some unusual properties of the DNA of a tumor-producing virus
Robert Weisberg	Biology Division, California Institute of Technology, Pasadena	Effects of polyoma virus in tissue culture
Gordon Whitmore	Ontario Cancer Institute, Toronto, Canada	Radiation induced mitotic delay in mammalian cells
F. T. Wolf	Department of Biology, Division of Molecular Biology, Vanderbilt University, Nashville, Tenn.	Some recent studies with plant pigments
Val Woodward	Department of Biology, Rice University, Houston, Tex.	Studies on PYR-3 mutants of Neurospora
D. L. Wulff	Institut für Genetik, Köln-Lindenthal, Germany	The role of thymine dimers in T2 phage inactivation

FOREIGN TRAVEL

During this report period 12 members of the Biology Division traveled to various parts of the world to attend and participate in meetings, present lectures and seminars, and consult with other scientists.

During March, M. A Bender went to Hiroshima, Japan, as consultant to the Atomic Bomb Casualty Commission. While he was in Japan he visited laboratories in Fukushima, Sapporo, Mishima, Nara, and Kyoto to discuss problems of mutual interest.

Also during March, Alexander Hollaender went to Geneva, Switzerland, to act as temporary adviser to the World Health Organization.

D. M. Prescott attended the meeting of the National Science Foundation Developmental Biology Panel held in Bimini, Bahama Islands, March 11–14.

R. C. von Borstel went to Athens, Greece, to present the paper "Effects of radiation on germ cells of insects: dominant lethals, gamete inactivation, and gonial cell killing" and to participate in discussions in a symposium on the Use and Application of Radioisotopes and Radiation in the Control of

Plant and Animal Insect Pests, April 22–26. This symposium was sponsored by the International Atomic Energy Agency and the Food and Agriculture Organization of the United Nations. Von Borstel also visited laboratories in Athens, Greece, and in Naples and Pavia, Italy. On May 6 he presented a seminar on "Radiation-induced dominant lethality in insects" at the International Laboratory of Genetics and Biophysics in Naples. On May 24 he spoke on "Spontaneous and induced mutations in yeast" at the Radium Institute in Paris, France.

In May, A. C. Upton, V. K. Jenkins, and C. C Congdon attended the 54th Annual Meeting of the American Association for Cancer Research held in Toronto, Canada. Dr. Upton attended the Board of Directors meeting and V. K. Jenkins presented a paper entitled "Transmission of radiogenic myeloid leukemia in the RF mouse" at the annual meeting.

W. E Cohn, who left the Division in September 1962 to spend a year abroad conducting research on viral nucleic acid problems, has lectured at laboratories and universities in England, Scotland, France, Germany, Switzerland, and Italy. His topics were "Determination of nucleotide structures," "New nucleotides," "New nucleotides, structure determination of nucleosides, and chromatography," and "Pseudouridine." In addition he presented a CERN Colloquium (Centre Européen de Recherches Nucléaire) titled "Determination of nucleotide structures" in June in Geneva, Switzerland. Dr. Cohn will return to the Division in September after visiting Israel, Thailand, Hong Kong, and Japan.

A. H. Haber attended the Fifth International Conference on Natural Plant Growth Regulators in Paris, France, from July 15–20. He presented a paper on "Interpretations concerning cell division and growth."

Six members of the Division attended the International Conference on Cell Division and Induction of Cancer by Radiation held in Lima, Peru, and Cali, Colombia, from July 1–6. In Lima (July 1), Joan Wright Goodman spoke on "Stem cells of the hemopoietic and lymphatic tissues"; on July 2, A. C. Upton on "Comparative studies on carcinogenesis by acute exposure to ionizing radiation." In Cali (July 5), D. M. Prescott spoke on "RNA and protein metabolism and intracellular migration during the cell life cycle." Alexander Hollaender presented the introductory and closing remarks at both the Lima and Cali sections of the conference and served as chairman of the morning session on July 5 (at Cali). C. C Congdon presented a summary of the meeting at the last session on July 6. W. J. Welshons, who is at the University of Chile, on leave of absence from the Division for a year, attended the meetings.

Before the conference, two Division members visited scientists in Peru and Chile. A. C. Upton went to Lima, Peru, to confer with Dr. Pablo Mori-Chavez about collaborative experiments on the influence of altitude on the development of radiogenic myeloid leukemia and other neoplasms in the RF mouse. He visited the high altitude laboratory at Marococha to observe the effects thus far on the animals there. Joan Wright Goodman went to the University of Chile in June to visit scientists there and to present a seminar on the "Origin and transplantation potentialities of peritoneal cells." Alexander Hollaender spoke at San Marcos University in Lima, Peru, on "Possibilities for cooperation between San Marcos University and the Biology Division, Oak Ridge National Laboratory, in biology."

Tuneo Yamada attended the Sixth International Embryological Conference held in Helsinki, Finland, July 22–25. He presented a paper on "Synthesis of macromolecules in Wolffian lens regeneration," and participated in a symposium on Competence, speaking on "Competence and the pattern of macromolecular synthesis." A demonstration, prepared by F. J. Finamore and Tuneo Yamada, was also given at the conference. While in Europe, Dr. Yamada visited institutions in Switzerland, Germany, Sweden, Denmark, and Finland to confer with scientists on matters of mutual interest.

In August, M. A Bender attended a meeting sponsored by the Pan American Health Organization of the World Health Organization and the United States Atomic Energy Commission in Rio de Janeiro, Brazil. The meeting was for studies on high background radiation areas in Brazil. While in that country he visited institutions in Rio de Janeiro and São Paulo.

SEVENTEENTH ANNUAL BIOLOGY RESEARCH CONFERENCE

The 1964 Research Conference, sponsored by the Biology Division of Oak Ridge National Laboratory, will be held April 6–9 at the Mountain View Hotel, Gatlinburg, Tenn. The organizing committee, composed of E. Volkin, chairman, R. B. Setlow, R. F. Kimball, A. C. Upton, G. D. Novelli, and C. C Congdon, announced the following tentative program:

THE MOLECULAR ACTION OF MUTAGENIC AND CARCINOGENIC AGENTS

Monday, April 6

Morning

Chairman – Seymour Benzer, Department of Biological Sciences, Purdue University, Lafayette, Ind.

Mutagens and DNA structure – Leonard S. Lerman, Department of Biophysics, University of Colorado, Denver

Mutagenesis and genetic fine structure – John W. Drake, Department of Microbiology, University of Illinois, Urbana

Afternoon

Chairman – Gunther S. Stent, Virus Laboratory, University of California, Berkeley

Mutagenesis and chromosome structure – F. J. de Serres, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Structure of bacteriophage proteins – Sydney Brenner, Laboratory of Molecular Biology, University Postgraduate Medical School, Cambridge, England

Tuesday, April 7

Morning

Chairman – Adolf Wacker, Institut für Therapeutic Biochemistry, Universität Frankfurt, Frankfurt am Main, Germany

Physical changes and mutagenesis – R. B. Setlow, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Base analogues and radiation sensitivity – Henry S. Kaplan, Department of Radiology, Stanford University School of Medicine, Palo Alto, California

Afternoon

Open

Wednesday, April 8

Morning

Chairman – Charles E. Carter, Department of Pharmacology, Yale University Medical School, New Haven, Conn.

Electronic aspect of the interactions between the carcinogens and possible cellular sites of their activity – Bernard Pullman, Institut de Biologie Physico-Chimique, Université de Paris, Paris, France

Alkylating agents and carcinogenesis – Alexander Haddow, Chester Beatty Research Institute, London, England

Afternoon

Chairman – Louis Siminovitch, University of Toronto, Toronto, Canada

Studies on the molecular mechanism of hydrocarbon carcinogenesis – Charles Heidelberger, McArdle Memorial Laboratory, The University of Wisconsin, Madison, Wis.

Changes in patterns of nucleic acid and protein synthesis caused by a purine analogue – Hubert Chantrenne, Laboratorie de Chimie Biologique, Université Libre de Bruxelles, Bruxelles, Belgium

Thursday, April 9

Morning

Chairman – Renato Dulbecco, Division of Biology, California Institute of Technology, Pasadena

Origin and nature of spontaneous mutations – G. E. Magni, Istituto di Genetica, Università di Pavia, Pavia, Italy

Viruses and carcinogenesis – Harry Rubin, Virus Laboratory, University of California, Berkeley

Summary – Renato Dulbecco

1963 BIOLOGY SYMPOSIUM

The Proceedings of the Sixteenth Annual Biology Symposium, *Macromolecular Aspects of the Cell Cycle*, held in April at Gatlinburg, Tenn., are being published as a supplement to the *Journal of Cellular and Comparative Physiology* and will be available in October 1963.

BIOMEDICAL DIRECTORS MEETING

Two meetings of the Biomedical Program Directors of the Division of Biology and Medicine, United States Atomic Energy Commission, were attended by members of the Biology Division. The February meeting, held at the University of Utah, Salt Lake City, was attended by C. C Congdon. The April meeting, held at Western Reserve University, Cleveland, Ohio, was attended by S. F. Carson and D. G. Doherty.

BONE MARROW CONFERENCES

Bone marrow transplantation and chemical protection conferences were first held in 1957 in Oak Ridge. These conferences provide a means for prompt exchange of the latest experimental information between the clinical and animal researchers. In addition to the Biology Division staff, many people and organizations participate in arranging for these meetings.

Proceedings of the meetings usually are published in *Blood, The Journal of Hematology*, by the Biology Division or by the sponsoring organization.

Biology Division members took active part in a round table discussion on *Bone Marrow Transplantation and Irradiation Protection* in Atlantic City, New Jersey, on April 16. Two papers were presented by Division members; one by W. H. McArthur, A. L. Kretchmar, and C. C Congdon, and the other by A. L. Kretchmar and C. C Congdon.

Other meetings being planned are:

November 22–23, 1963, Oak Ridge, Tenn. – *Protection and Recovery Conference.* The three sessions and their chairmen are:

Chemical Protection and Related Topics – Chairman, Raymond Shapira

Bone Marrow Transplantation and Related Topics – Chairman, Daniel Billen

Gene Control of Cell Division – Chairman, Howard Adler

September 7–9, 1964, Paris, France. – Conference, tentative topic – *Bone Marrow Transplantation and Secondary Disease.*

HABROBRACON GENETICS RESEARCH CONFERENCE

Sixteen scientists from the United States gathered at the Biology Division on February 22–23 to participate in a Habrobracon Genetics Research Conference, the first of its kind ever held. Two members of the Biology Division took part in the informal conference. These were R. C. von Borstel and Gladys S. Van Pelt.

The purpose of the conference was to discuss problems relating to the Habrobracon Stock Center and to exchange information on methods for culturing and handling the wasp and its host. Current research projects on the wasps *Habrobracon, Mormoniella,* and *Nemeritis* were discussed in detail by those present. It is expected that this conference will be held at two- or three-year intervals.

Dr. R. C. von Borstel organized the conference.

SPEAKERS AT PROFESSIONAL MEETINGS, FALL–WINTER 1963

Cryogenic Engineering Conference – Peter Mazur

Gordon Research Conference on "Separation and Purification" – C. L. Burger

Gatersleben Symposium on Genetics – Drew Schwartz

Fifteenth Autumn Meeting of The American Physiological Society – W. D. Fisher (coauthors, G. B. Cline and N. G. Anderson) and T. P. McDonald (coauthor, T. T. Odell, Jr.)

Fourteenth Annual Meeting of American Institute of Biological Sciences – H. I. Adler, B. M. Cattanach, G. Cudkowicz (coauthor, J. H. Stimpfling), T. J. Long (coauthor, A. H. Haber), Rusty J. Mans, (coauthors, Caridad Maria Purcell and G. D. Novelli), C. A. Price (coauthors, N. G. Anderson, W. D. Fisher, R. E. Canning, and C. L. Burger), Liane Brauch Russell (coauthors, M. N. Cupp McDaniel and F. N. Woodiel), Frank Seto, H. E. Walburg, Jr. (coauthor, G. Cudkowicz), Sheldon Wolff (coauthor, H. E. Luippold), and Florence N. Woodiel (coauthor, Liane Brauch Russell)

Eleventh International Congress of Genetics – Sandra Bell (coauthor, Sheldon Wolff), M. A Bender (coauthor, P. C. Gooch), Ernest H. Y. Chu, Gale Davis, F. J. de Serres, E. H. Grell, Rhoda F. Grell, R. F. Kimball, E. F. Oakberg, Liane Brauch Russell, W. L. Russell, R. E. Scossiroli (coauthor, R. C. von Borstel), R. C. von Borstel, W. J. Welshons (coauthors, E. S. Von Halle and B. J. Scandlyn), and Sheldon Wolff (coauthor, F. J. de Serres)

Eighteenth Annual Meeting of Society of General Physiologists – R. B. Setlow (coauthors, P. A. Swenson and W. L. Carrier)

145th National Meeting of American Chemical Society – F. J. Finamore (coauthor, T. Yamada)

International Symposium on Physiology, Ecology, and Biochemistry of Germination – A. H. Haber

Second Mammalian Chromosome Conference – M. A Bender (coauthor, P. Carolyn Gooch), E. H. Y. Chu (coauthors, T. C. Hsu and D. E. Norby), and P. Carolyn Gooch (coauthor, M. A Bender)

Symposium on "Recent Developments in Research Methods and Instrumentation" – M. L. Randolph

International Atomic Energy Agency Symposium on "Biological Effects of Neutron Irradiations" – P. Carolyn Gooch (coauthors, M. A Bender and M. L. Randolph) and A. C. Upton (coauthors, M. L. Randolph, K. W. Christenberry, E. B. Darden, Jr., and J. W. Conklin)

Third Annual Meeting of The American Society for Cell Biology – C. L. Burger (coauthors, N. G. Anderson and W. W. Harris), Ivan L. Cameron, O. L. Miller, Jr., George M. Padilla (coauthor, P. A. Van Dreal), R. M. Ritossa (coauthor, J. F. Pulitzer), G. E. Stone (coauthor, D. M. Prescott), R. C. von Borstel (coauthors, D. M. Prescott and F. J. Bollum), and D. M. Prescott, program chairman

Southeastern Regional Meeting of the American Chemical Society – R. H. Dinius (coauthors, M. L. Randolph, J. S. Kirby-Smith, and R. R. Becker) and F. T. Kenney

EDUCATION

Student Trainee Program. – This year 17 juniors from small colleges in 12 states, representing all sections of the United States, were selected on a competitive basis to serve on temporary summer appointments in the Biology Division. This Summer Student Trainee Program is supported by the United States Atomic Energy Commission and administered by the Oak Ridge Institute of Nuclear Studies in cooperation with Oak Ridge National Laboratory.

Through this Program, specially selected juniors from small colleges, primarily in the southern region, are assigned to and trained within the Biology Division. They are provided the atmosphere and opportunity to develop a better comprehension of research methods and their significance by carrying out research activities under the guidance of Biology Division scientists.

Each student selects, plans, and executes a research project during his stay. Upon the completion of his project he prepares a paper describing the work and results.

The 1963 students, their affiliations, and the sections to which they were assigned were:

STUDENT TRAINEE	INSTITUTION	ASSIGNED TO
Boone, Charlotte M.	Converse College Spartanburg, S.C.	E. H. Y. Chu Mammalian Genetic Cytology
Breakefield, Xandra O.	Wilson College Chambersburg, Pa.	L. B. Russell Mammalian Genetics

STUDENT TRAINEE	INSTITUTION	ASSIGNED TO
Bredahl, Karen T.	Hood College Frederick, Md.	L. H. Smith Mammalian Recovery
Gall, Clara Lee	Rosary Hill College Buffalo, N.Y.	C. J. Wust Enzymology
Gladney, Gail M.	University of Chattanooga Chattanooga, Tenn.	M. A Bender Tissue Culture
Griffith, Jack D.	Occidental College Los Angeles, Calif.	R. B. Setlow Biophysics
Holland, Joanna	Westhampton College University of Richmond, Va.	E. F. Oakberg Drosophila Cytology and Genetics
Kirkpatrick, Barbara	Winthrop College Rock Hill, S.C.	F. J. Bollum Molecular Biology
Leskes, Andrea	Vassar College Poughkeepsie, N.Y.	J. K. Setlow Phage Genetics
Lowery, Bettye A.	Florida A & M University Tallahassee, Fla.	R. F. Kimball Effects of Radiation on Paramecium
Mather, John R.	Western Kentucky State College Bowling Green, Ky.	M. A. Kastenbaum Biometrics
Miller, Barbara A.	North Georgia College Dahlonega, Ga.	R. F. Grell Drosophila Cytology and Genetics
Oliver, Richard W.	Asbury College Wilmore, Ky.	S. Wolff Chromosome Cytology
Scurry, Carolyn	Winthrop College Rock Hill, S.C.	A. H. Haber Plant Physiology
Smith, Channing D.	Virginia Union University Richmond, Va.	E. H. Grell Drosophila Cytology and Genetics
Stewart, Marjorie A.	Winthrop College Rock Hill, S.C.	C. C Congdon Mammalian Recovery
Tullos, Nelwyn J.	Northeast Louisiana State College Monroe, La.	W. D. Gude Pathology and Physiology

Also, three other students who were not under the Student Trainee Program were selected to train in the same manner described above. They were:

STUDENT	INSTITUTION	ASSIGNED TO
Albritton, William L.	University of Alabama Tuscaloosa, Ala.	G. D. Novelli Enzymology
Berger, James D.	Indiana University Bloomington, Ind.	R. F. Kimball Effects of Radiation on Paramecium
Kendrick, Joseph	Knoxville College Knoxville, Tenn.	C. C Congdon Mammalian Recovery

This program, now in its sixth year, has been instrumental in encouraging undergraduate students to continue on with graduate work.

This year a new program was instituted for students who had completed their undergraduate work and had been accepted for graduate school. Five Student Trainees of 1962 were here during the summer under this new program. These Senior Trainees, their undergraduate schools, the graduate schools in which they are enrolled for 1963, and the sections in the Biology Division to which they were assigned were:

SENIOR STUDENT TRAINEE	UNDERGRADUATE SCHOOL	GRADUATE SCHOOL	ASSIGNED TO
Barbee, Barbara A.	Centenary College of Louisiana, Shreveport, La.	Purdue University	E. H. Y. Chu, Mammalian Genetic Cytology
Groeniger, Eleanor	Mt. Holyoke College, South Hadley, Mass.	Johns Hopkins University, Baltimore, Md.	F. J. Bollum, Molecular Biology
Joyner, Julia A.	Winthrop College, Rock Hill, S.C.	University of Wisconsin, Madison, Wis.	L. B. Russell, Mammalian Genetics
Lorick, Gloria C.	Winthrop College, Rock Hill, S.C.	University of Rochester, Rochester, N.Y.	D. M. Prescott, Cell Growth and Reproduction
Webb, Brenda C.	Pfeiffer College, South Hadley, Mass.	Western Reserve University	R. A. Popp, Mammalian Biochemical Genetics

Small College Program. – The Small College Program, set up by the Biology Division, is basically an effort by the Division to offer assistance in the form of lectures to small colleges. Two Division members participated in the program during this report period: D. G. Doherty spoke on "Enzymes, the biological catalysts" at Spring Hill College, Spring Hill, Alabama, on April 29. R. F. Kimball spoke on "Intraclonal genetics of *Paramecium tetrahymena*" at Longwood College, Farmville, Virginia, on May 10.

University Cooperative Program. – Two Biology Division members gave three lectures at the University of Georgia, Athens, as part of the cooperative graduate and postdoctoral teaching program between that University and the Division: R. J. Mans spoke to the Biochemistry Department on "The evolution of an amino acid incorporating system from maize" on February 15. S. F. Carson spoke to the Bacteriology Department on the "Relation between growth of bacteria and ATP generation" on May 23 and on the "Theoretical energy requirements for growth of bacteria" on May 24.

Courses. – Eight Biology Division members participated in four courses during this report period.

W. L. Russell spoke on "Genetic effects" as part of a course in Radiological Biophysics at the University of California Division of Medical Physics, Berkeley, on May 1.

D. M. Prescott presented a course in tissue culture at the University of Wisconsin, Madison, on June 23–24.

Five members of the Biology Division took part in the U.S. Army Nuclear Science Seminar held at ORINS on August 19. Speakers and titles were:

W. D. Gude	"Basic radiobiology"
E. B. Darden	"Effects of radiation on biological organisms"
T. T. Odell, Jr.	"Biological effects of radiation on mammals"
E. F. Oakberg	"Genetic effects of radiation"
L. H. Smith	"Radiation protective and recovery measures"

Those who attended the seminar were given a tour of the Biology Division on August 20.

G. E. Cosgrove went to Wayne State University, Detroit (July 24–28), and Florida State University, Tallahassee (July 28–31), to give lectures and demonstrations in mammalian radiobiology to students in the NSF-AEC Summer Institute in Radiation Biology for Science Teachers.

Training Programs. – Eleven Biology Division members gave 11 lectures in training programs and institutes during this period.

Four members participated in the 13th Session, Oak Ridge Science Lecture Demonstration Program given by ORINS:

Adler, H. I.	"Environments that permit life"	March 19
Anderson, N. G.	"New methods of virus isolation"	March 20
Jagger, J.	"Ultraviolet radiation and the origin of life"	March 25
Popp, R. A.	"The effects of radiation on mammals"	March 26

Six members lectured at the Oak Ridge School of Reactor Technology during May:

Kimball, R. F.	"Cellular aspects of radiation damage"	May 20
Stapleton, G. E.	"Protection and recovery in microorganisms"	May 22
Congdon, C. C	"Protection and recovery in mammals"	May 24
Oakberg, E. F.	"Effects of radiation on mammalian germ cells"	May 27
Russell, L. B.	"Genetic effects of radiation on mammals"	May 29
Cosgrove, G. E.	"Acute effects of radiation on mammals"	May 31

J. F. Albright gave a lecture on "Some dynamic aspects of antibody production" as part of the Embryology Training Program sponsored by Carnegie Institution of Washington at Woods Hole, Mass., on June 29.

COURSES ATTENDED

G. E. Cosgrove attended the Summer Program in Acarology at the Institute of Acarology at Ohio State University, Columbus, Ohio, from June 17 to July 6.

D. A. Fuccillo, Jr., attended the Sixth Annual Institute in Technical and Industrial Communications from July 8–12 at Colorado State University, Fort Collins.

DIVISION MEMBERS ON LEAVE

W. E Cohn left the Division in September of 1962 on leave of absence to spend a year abroad conducting research on viral nucleic acid problems. He worked at the Agricultural Research Council, Virus Research Unit, Cambridge, England, and at the Institute of Biology, Paris, France, under grants from the Guggenheim Foundation and the National Institutes of Health. Dr. Cohn will visit laboratories in Europe and Japan before returning to the Division in September.

J. S. Kirby-Smith has been on leave of absence with the Division of Biology and Medicine of the Atomic Energy Commission, Washington, D.C., since January.

D. R. Krieg is on leave of absence from the Division and is with the Department of Chemistry, Stanford University, Palo Alto, California, working under a special United States Public Health Service fellowship.

D. L. Lindsley left June 2 to spend the summer in the Department of Genetics, University of Washington, Seattle. He will return on September 1.

W. A. Arnold left June 15 for the Marine Biological Laboratory, Woods Hole, Massachusetts, where he is carrying on research on energy transfer in biological systems and consulting with other scientists engaged in related studies. He will return September 15.

W. J. Welshons left the Division in February to work one year at the University of Chile, Santiago, under a National Science Foundation Senior Postdoctoral fellowship.

R. A. Wallace left in August to spend 10 months in Ottawa, Canada, at the National Research Council in collaborative work between the Biology Division and the Council's Division of Applied Biology.

Cytology and Genetics

Effects of Radiation on Paramecium		Chromosome Cytology	
R. F. Kimball	E. G. Bailiff	Sheldon Wolff	H. E. Luippold
J. O. Krivanek[a]	Stella W. Perdue	Sandra Bell[f]	Judith M. Pier
D. C. Shepard[b]	H. Marine Scandlyn	J. D. Caponetti[a]	R. W. Oliver[d]
	Carrie L. Wells	T. H. Ma[a]	
	J. D. Berger[c]	Sister Augustine Mattingly[a]	
	Bettye A. Lowery[d]		
Phage Genetics		**Fungal Genetics**	
D. R. Krieg[e]	Maxon E. Boling	F. J. de Serres	Anita M. Meley
Jane K. Setlow	Andrea Leskes[d]	B. B. Webber	Ida C. Miller
C. M. Steinberg	Ursula B. Steinberg[f]	W. E. Barnett[g]	Arlee P. Teasley
		H. E. Brockman[g]	
[a]Research participant.		**Mammalian Genetic Cytology**	
[b]National Science Foundation Fellow.		E. H. Y. Chu	Lana T. Covington
[c]Temporary summer employee.		Tien-Ding Chang[h]	Patricia G. Lankford
[d]Student trainee.		Margaret Y. Menzel[i]	Nancy H. Robinson
[e]Leave of absence, Stanford University.			Barbara A. Barbee[c]
[f]Consultant.			Charlotte M. Boone[d]
[g]USPHS Fellow.		**Mammalian Biochemical Genetics**	
[h]Visiting investigator from abroad.		R. A. Popp	Diana M. Popp
[i]Loanee.			Brenda C. Webb[c]

DISTRIBUTION OF MUTATION TO CONSERVED STRANDS IN PARAMECIUM

R. F. Kimball H. Marine Scandlyn
Stella W. Perdue E. G. Bailiff

Introduction. – The distribution of mutations among the daughter cells of a treated cell can yield considerable information about the initial steps in mutagenesis. It has been shown for a number of cells[1] that (1) the preduplication chromosomes consist of two conserved strands, (2) two new strands are made at duplication, and (3) at the subsequent division the daughter cells each get one old and one new strand. At the second division the initial old strand and the associated new one of the first generation segregate from one another. Thus the four strands of

[1]See, for review, J. H. Taylor in *Molecular Genetics*, Part 1 (ed. by J. H. Taylor), p 65, Academic Press, New York, 1963.

the first cell generation, the two old and two new ones, should each be segregated to a different product of the second division; and the distribution of a mutation among these products should tell us which strands were affected.

Method. – Dividing paramecia were individually isolated, and one daughter cell was exposed during preduplication interphase, either to 500 r of x rays or 640 ergs/mm^2 incident intensity of 2537 A ultraviolet; the other daughter was kept as an unirradiated control. At each of the next three divisions in both the irradiated and control lines of descent, the daughter cells were isolated into separate containers to give a total of 16 pedigreed isolates, 8 irradiated and 8 control. These 16 isolates were allowed to multiply by division, autogamy was induced in the resulting cultures, 25 autogamous specimens were isolated from each culture, and the presence or absence of mutation was judged from the presence or absence (above the control level) of autogamous isolates that grew poorly or died. The individual controls made it possible to eliminate those cases in which spontaneous mutation occurred in cell generations prior to the treated one.

Results. – In all except four of the ultraviolet cases, the two products of the third division were concordant for induced mutation as expected if the only relevant strands were those present at some time during the irradiated cell generation. The five possible distributions of mutation to the products of the second division are: all four products mutant (whole); the two products from one product of the first division mutant (half); one product of the second division mutant (quarter); and the combinations, one-half and one-quarter and double quarters. Table 1 shows that only the first two of these were found, with the exception of a single quarter in the x-ray group. This latter was probably a spontaneous event since an equal frequency of quarters was found in the control. In addition, 17% of the lines in both the x-ray and ultraviolet groups could not be used because of spontaneous events occurring before treatment, as judged by high frequencies of slow growth and death in both the irradiated and control groups.

The x-ray exposure was low enough to produce few, if any, cases of more than one mutation in the same individual; but the ultraviolet exposure was too high and must have produced a considerable frequency of multiple mutations. Thus the x-ray data can be taken at their face value to show that 80 to 90% of the mutations were wholes. Part of the apparent wholes in the ultraviolet group, however, must have been multiple halves since mutations in different chromosomes would segregate independently to the two halves. When this is taken into account by an appropriate formula, half mutations can be calculated to comprise about 40% of the total.

Table 1. Distribution of Mutations in *Paramecium*

Classification[a]	Number of Cases	
	X Rays	Ultraviolet
Whole	13	14
Half	2	4
Quarter	1	0
None	55	7
Unusable (high control)	15	5
Total	86	30

[a]See text.

Discussion. – Under the conditions of the experiment a large fraction of the x-ray mutants should have arisen from reparable lesions that were fixed as final mutation only when the chromosomes duplicated.[2] The present data show that the initial reparable lesion must affect both old strands and that the lesion, though reparable before duplication, must become irreparable at duplication; otherwise some of the old strands should have been repaired after producing mutant new strands, thus producing single or double quarters. Many events, alterations of single DNA bases for example, would not appear to have the requisite properties. Among known events, double DNA chain breaks, held in juxtaposition by chromosomal protein up to duplication but pulled apart during synthesis, seem to come closest to having the required properties.

[2]See, for review, R. F. Kimball, *J. Cellular Comp. Physiol. Suppl. 1*, **58**, 163 (1961).

SPECIFIC INCORPORATION OF DNA PRECURSORS BY FEEDING LABELED BACTERIA TO *PARAMECIUM AURELIA*

J. D. Berger R. F. Kimball

Introduction and Methods. – A number of studies have been made on the incorporation of exogenous DNA, RNA, and protein precursors into *Paramecium aurelia*. This organism usually derives its metabolites from bacteria. Therefore, it seemed possible that the exogenous route was less efficient and possibly less specific than the normal bacterial route. Specifically, Kimball and Perdue[3] have found that exogenous tritiated thymidine at 100 μc/ml is incorporated into the macronuclear DNA but also appears in the cytoplasm after 10 to 15 min. It is possible that the mutagenic analog of thymidine, bromodeoxyuridine (BUdR), would have the same fate. Therefore experiments were undertaken to introduce these substances by the normal bacterial route by feeding bacteria which had incorporated these compounds.

A thymine-requiring strain of *Escherichia coli*, 15 T^-, was grown for approximately one cell generation in the presence of exogenous isotopically labeled DNA precursors, either methyl H^3-thymidine or bromouracil-2-C^{14}, washed free of exogenous label and fed to paramecia at known stages in the life cycle. The paramecia were dried on slides and fixed, and autoradiographs were prepared.

Results and Discussion. – Tritiated thymidine derived from the digestion of labeled bacteria was specifically incorporated into the macronucleus, the cytoplasm remaining free of label, while exogenously labeled control animals had label over both nucleus and cytoplasm (see Table 2). Bromouracil, C^{14}-labeled, also is incorporated by the bacterial route only into DNA, as shown by digestion of labeled cells with DNase and RNase.

It was found that activity appears in food vacuoles very soon after the paramecia are placed in the suspension of labeled bacteria, as many as four or five labeled vacuoles forming within 5 min from the start of labeling. The lifetimes of vacuoles seem to be over 2 hr, with some lasting 3 hr or more.

Table 2. Comparison of Grain Counts over Nucleus and Cytoplasm of Exogenously and Bacterially Labeled Paramecia

Type of Labeling	Grains per Unit Area	
	Nucleus	Cytoplasm
Exogenous	27.8	12.2
Bacterial	34.6	0.3

Labeled thymidine derived from bacteria is apparently incorporated into the macronucleus at a constant rate during the macronuclear synthetic period, but there appears to be a short lag of about 8 min after the animals are placed in the suspension of labeled bacteria before incorporation begins. Data suggest that the macronuclear label is conserved, but some details are still unclear.

Some evidence has been found for the existence of a soluble pool of DNA precursors by applying wet photographic emulsion to unfixed animals. Data suggest conservation of part of this pool, but details are as yet uncertain.

It was hoped that the specificity of this method of introducing DNA precursors would make possible the induction of appreciable amounts of mutation by BUdR. Unfortunately it was found that, even when paramecia were in a suspension of BUdR-containing bacteria for as long as two cell cycles, no significant amount of mutation occurred. Since the compound is known to be specifically incorporated into DNA, by the autoradiographic studies, the results suggest a very low mutagenic efficiency in this organism.

Conclusions. – It has been shown that DNA precursors taken up by *P. aurelia* through the digestion of isotopically labeled bacteria are specifically incorporated into DNA. It is hoped that the specificity of this technique for the incorporation of DNA precursors may make it usable for the study of a variety of problems concerning uptake, distribution, and conservation of metabolites in this and other bacterial feeding organisms.

[3]R. F. Kimball and Stella W. Perdue, *Exptl. Cell Res.* 27, 405 (1962).

UPTAKE OF THE AMINO ACID H^3-LEUCINE BY *PARAMECIUM AURELIA*

Bettye A. Lowery R. F. Kimball

Introduction. – Earlier microspectrophotometric and microinterferometric measurements of *Paramecium aurelia,* syngen 4, stock 51 (ref 4), established that the dry weight and so presumably the total protein of the whole paramecium increased approximately exponentially during one cell generation. Total dry weight, however, measures unincorporated material in the food vacuoles as well as incorporated material in the cytoplasm and nucleus. The rate of formation of new protein, in theory, could be determined by the rate of incorporation of amino acids. The present experiments were designed to measure this incorporation through the cell cycle using H^3-leucine and quantitative autoradiography.

Method. – *P. aurelia,* syngen 7, stock 51, lacking kappa was grown in lettuce infusion containing *Aerobacter aerogenes.* Dividing paramecia were collected and washed twice to dilute the bacterial population, and H^3-leucine was added in sterile lettuce infusion. After 25 to 30 min the paramecia were washed, dried on slides, fixed, and covered with NTB-3 liquid emulsion. After the autoradiographs were developed, the grains were counted over a unit area of cytoplasm as a measure of the rate of incorporation per unit weight.

Results. – The data are plotted in Fig. 1. The rate of incorporation, as measured by the number of grains per unit area, reaches a maximum at 3 hr after division and declines again to reach a minimum at division.

Discussion. – The curve in Fig. 1 differs significantly from expectations, with either an exponential or linear increase in total protein. With pulse labeling, grains per unit area should be proportional to the rate of increase in protein per unit dry weight. This quantity should be a constant throughout the cell cycle if total protein and dry weight were increasing exponentially; it should decrease continuously if total protein were

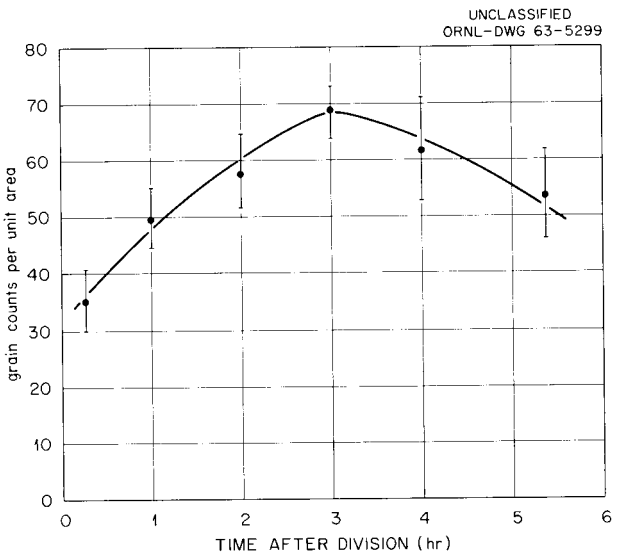


Fig. 1. Grains per Unit Area After Uptake of H^3-Leucine. The count was for a 25- to 30-min period, starting at the time after division shown on the abscissa. The last point represents specimens which divided while in isotope; 95% confidence limits are given.

increasing linearly. The actual curve, though differing significantly from either of these expectations, can be shown to differ from an exponential by only a few percent at any time during the cell cycle. This difference could not be detected by any direct measurement of total protein or dry weight, though it is very obvious with the autoradiographic method. The autoradiographic method measures only cytoplasmic, not food vacuole, protein. Consequently, the contribution of food vacuole dry mass to the earlier dry weight measurements could not have changed the form of the curve very much; otherwise the agreement of the two methods would not have been as good as it was.

There is still a possibility that the true form of the curve is exponential and that the incorporation curve has been distorted by variations in pool size or rate of uptake of the exogenous amino acid. If this is not so, however, then the curve shows that the rate of protein synthesis per unit mass varies through the cycle, reaching a maximum near the midpoint.

[4]R. F. Kimball *et al., Exptl. Cell Res.* 17, 160 (1959).

MODEL SYSTEM FOR INVESTIGATION OF MUTAGEN-INDUCED DNA REPLICATION ERRORS

D. R. Krieg

Introduction. – Hydroxylamine treatment of extracellular virus particles or genetically active DNA induces point mutations. Such mutations are believed to arise during duplication of nucleic acid containing a base that has been altered by hydroxylamine and to consist of the substitution of one base at the site of another. The DNA constituent with which hydroxylamine reacts most rapidly is cytosine, and one or more of the known cytosine reaction products can be presumed to account for the induced mutations. From the patterns of mutations induced by hydroxylamine and base analogs, it has been postulated that the hydroxylamine-altered cytosine directs the incorporation of adenine to substitute for its normal guanine pairing partner. A preliminary experiment is described here which is designed as a direct test of this hypothesis; it utilizes an *in vitro* system which should be very useful as a model system for investigating mutagen-induced, base-pair substitutions. Analogous experiments with 5-bromouracil were reported by Trautner *et al.*[5]

Experimental. – The DNA polymerase obtained from *Escherichia coli* can catalyze the polymerization of synthetic polynucleotides and can utilize these as templates for the synthesis of complementary polymers. One such polynucleotide, polydeoxycytidine (poly dC) was used in this experiment. Polymerization reaction mixtures contained hydroxylamine-treated or control poly dC as template and dGTP and dATP as substrates. Polymerization was observed in parallel reaction mixtures, each containing one of the substrates labeled with P^{32} in the position proximal to the sugar, and the amount of nucleotide incorporation into polymer occurring in standard conditions during a 1-hr incubation was measured by perchloric acid–precipitable radioisotope. The hydroxylamine treatment was for 5 hr with 1 *M* NH_2OH, phosphate buffered at pH 7.4. The mixture was divided into three portions for treatment at 50, 40, and 0°C, respectively, the latter serving as a control. The treatment was terminated by addition of acetone and by icing; then each portion was dialyzed at 4°C, pH 7.4, ultimately against 0.01 *M* phosphate buffer. Aliquots of these preparations and of an untreated poly dC, as an additional control, were used as templates in the polymerization reaction mixtures. The results are presented in Table 3.

The figures tabulated for $dAMP^{32}$ incorporated are a maximal estimate; they are corrected for counter background but not for a small amount of radioisotope (0.00004 of the input) found in the acid precipitate of a control reaction mixture containing treated poly dC and complete except for polymerase. Since this amount of radioisotope was only one-seventh the amount found in the parallel reaction mixture complete with polymerase, the results are regarded as showing significant hydroxylamine treatment-directed incorporation of dAMP. However, the incorporation of dAMP tabulated for the two control polymerizations with untreated poly dC may not be significant.

Discussion. – Although it is suggestive, this experiment cannot yet be regarded as proof that a hydroxylamine-produced cytosine product in a polynucleotide directs the incorporation of an adenine in the place of a guanine. A crucial test will be to digest enzymatically the polymer produced under these conditions to 5′-mononucleotides and to 3′-mononucleotides and to analyze the radioactivity of the components. The first digestion could confirm that the acid-precipitated P^{32} truly represents dAMP incorporation and not, for instance, a hypothetical contaminant of the $dATP^{32}$ preparation. The second digestion could test whether the incorporated dAMP is polymerized next to dGMP as single replication errors and not, for instance, as a sequence of adenine nucleotides. Also, it remains to be determined whether or not the incorporation of nucleotides other than adenine may be directed by hydroxylamine treatment of poly dC. It should also be possible to investigate whether the replication error is an irrevocable result of the template properties of the altered cytosine or whether incorporation of adenine and guanine are alternatives which can be influenced by substrate concentrations or other variables.[6]

[5] T. A. Trautner, M. N. Swartz, and Arthur Kornberg, *Proc. Natl. Acad. Sci. U.S.* **48**, 449 (1962).

[6] This work was performed while the author was a USPHS Fellow at Stanford University Department of Biochemistry. He gratefully acknowledges the technical advice and material assistance of the members of that department.

Table 3. Nucleotide Incorporation into Polymers Synthesized with Hydroxylamine-Treated Poly dC Template

	Treated			Untreated
	50°C	40°C	0°C	
Ratio of $dGMP^{32}$ incorporated to amount of input poly dC	0.009	0.040	0.072	0.075
Ratio of $dAMP^{32}$ incorporated to $dGMP^{32}$ incorporated	0.0059	0.0021	0.0003	0.0003

ACTION SPECTRUM OF AN *IN VITRO* DNA PHOTOREACTIVATION SYSTEM

Jane K. Setlow Maxon E. Boling

Introduction. – It is known that ultraviolet-inactivated transforming DNA may be partially reactivated by exposure to light in the presence of a cell-free yeast preparation. Most or all of the biological effect of the yeast extract may be attributed to only one photochemical reaction: the splitting of ultraviolet-induced thymine dimers in the DNA.[7] The wavelength dependence of *in vitro* photoreactivation by yeast extract has been determined in the hope that the shape of the action spectrum might be a clue to the identity of the light-absorbing compound responsible for the reaction.

Results and Discussion. – To determine the efficiency of photoreactivation by light of different wavelengths, we should consider the kinetics of the reaction. There is an intensity-dependent lag at the beginning of the biological reaction. That there is a similar lag in the splitting of thymine dimers in the DNA has been shown by corresponding chemical assays. After the lag period the increase in biological activity of the DNA is approximately linear with time of illumination, until a plateau is reached. The height of the plateau, representing maximum photoreactivation, is approximately the same, regardless of wavelength and intensity of illumination. The slope of the linear portion of the curve was used as the measure of photoreactivation rate. The efficiency of photoreactivation at a given wavelength was taken to be the rate at an arbitrary intensity, low enough so that at all wavelengths it was in a range of intensities where rate and intensity were linearly related.

The most efficient wavelengths for photoreactivation are about 3550 and 3850 A. Although the action spectrum is not very similar to flavin absorption, riboflavin at very low concentration inhibits photoreactivation, as it also inhibits a number of flavoenzymes, suggesting that the photoreactivating enzyme might be a flavoprotein.

[7] Jane K. Setlow and R. B. Setlow, *Nature* **197**, 560 (1963).

METHYLENE BLUE INHIBITION OF AN *IN VITRO* DNA PHOTOREACTIVATION SYSTEM

Andrea Leskes Jane K. Setlow

Introduction. – It is sometimes possible to gain information about the mechanism of a reaction by investigating the way in which certain inhibitors react. Two possible photoreactivation inhibitors have been tested, because they are inhibitors of some flavoenzymes, and there is a little evidence that the yeast photoreactivating enzyme could be a flavoenzyme.

Results and Discussion. – Caffeine was found to have no effect on *in vitro* photoreactivation of transforming DNA, but methylene blue has a large effect, in that the rate is decreased markedly by 0.8 μM methylene blue. This inhibitory effect cannot be explained in terms of photodynamic action of methylene blue on either the enzyme or the DNA, since at the methylene blue concentrations used, neither the enzyme nor the DNA is appreciably inactivated.

Methylene blue could inhibit in three possible ways: (1) noncompetitively, (2) competitively by combining with the enzyme, and (3) competitively

by combining with the substrate (ultraviolet lesions in the DNA). It is possible to distinguish between these three by investigating the kinetics of the inhibition. The substrate (S) may be varied by changing the concentration of the inactivated DNA. For the purpose of making a Lineweaver-Burk plot (a graph of reciprocal of the rate vs reciprocal of the substrate concentration), the rate v should be in terms of number of lesions repaired per unit time. Since ultraviolet dose is linearly related to number of lesions, v is proportional to dose reduction for any one concentration of DNA. The relative rate at different DNA concentrations may be normalized by multiplying dose reduction by DNA concentration.

Over a DNA concentration range of 10^3, the reciprocal plots yield straight lines (from data taken with and without the inhibitor present), which intersect at $1/S = 0$. This suggests that methylene blue inhibits competitively, probably by combining with enzyme rather than with the DNA, since substrate binding inhibition yields a curved line on the Lineweaver-Burk plot. Furthermore, the data make it possible that photoreactivation is an oxidation-reduction reaction, since methylene blue (which itself forms an electromotively active oxidation-reduction system) could be a competitor in such a reaction.

PHOTOREACTIVATION AND PHOTODYNAMIC INACTIVATION OF DNA

Andrea Leskes Jane K. Setlow

Introduction. – Proteins and nucleic acids may be altered by exposing them to visible light in the presence of certain dyes. Guanine is preferentially destroyed when DNA is illuminated with methylene blue.[8] It is of interest to determine whether methylene blue plus light produces lesions in transforming DNA which are photoreactivable and whether this photodynamically inactivated DNA will compete with ultraviolet (uv)-inactivated DNA for the photoreactivation enzyme in yeast, especially since a uv-irradiated polymer containing only the bases guanine and cytosine has been shown to inhibit photoreactivation of uv-irradiated transforming DNA.[9]

Results and Discussion. – No reversal of the methylene-blue-induced inactivation of transforming DNA was obtained when the DNA was illuminated in the presence of yeast enzyme. Inactivation of the photoreactivating enzyme by the methylene blue which was present in the DNA solution could be ruled out as the explanation for the lack of photoreactivation, since irradiated DNA added to the mixture could be photoreactivated. It was concluded that methylene-blue-inactivated DNA did not compete with irradiated DNA for the yeast enzyme, since the kinetics of the increase in the transforming ability of the irradiated DNA were not changed as a result of the presence of photodynamically inactivated DNA. These data show that (1) the uv lesion in poly G-C is different from the guanine damage in DNA caused by methylene blue, and (2) the photodynamically inactivated DNA has no specificity for the yeast enzyme and will not photoreactivate.

GENETIC DRIFT IN PHAGE-INFECTED CELLS

C. M. Steinberg

Introduction. – A bacteriophage-infected cell contains a small population of so-called "vegetative phage." These vegetative phage replicate, mutate, recombine, and become transformed into mature phage particles – all more or less simultaneously. The population dynamics of the infected cell can be an important factor when any of these processes are studied. We have previously given a theoretical treatment of the distribution of spontaneous mutants among phage-infected cells.[10] This treatment, which assumed a simple model of the kinetics of phage growth and maturation, was quite successful in explaining the experimentally observed distribution of mutants and, in addition, was able to predict quantitatively the large observed variance of total phage yields from single cells.

We have also given a treatment of the kinetics of recombination of phage.[11] This is a more difficult problem, since recombination, unlike mutation, involves the interaction of two or more

[8] M. I. Simon and H. van Vunakis, *J. Mol. Biol.* **4**, 488 (1962).

[9] C. S. Rupert, *J. Cellular Comp. Physiol. Suppl. 1*, **58**, 56 (1961).

[10] C. Steinberg and F. Stahl, *J. Theoretical Biol.* **1**, 488 (1961).

[11] C. Steinberg and F. Stahl, *Cold Spring Harbor Symposia Quant. Biol.* **23**, 42 (1958).

phage, and the treatment assumed that the population size within the infected cell was effectively infinite. Recent downward revisions in estimates of the population size call this assumption into question, and the possibility arises that some unexplained interference phenomena could be consequences of the small population size, that is, could be due to "genetic drift."

Results. – We have succeeded in writing the relevant equations using the same simple model that was used in the treatment of the mutant distribution. The solution of these equations has been achieved, however, only for the limiting case of very small map distances. The solution can be written as

$$\frac{dR}{dt} = 2a(1-a)\,p\left(1 - \frac{2t}{m} + \frac{2t^2}{m^2}\right) - 2a(1-a)\,p\exp\left(-\frac{m}{1+t}\right)\left[\frac{1}{(1+t)^2} - \frac{2t}{m(1+t)} + \frac{2t^2}{m^2}\right],$$

where

R = frequency of recombinants,

t = time,

m = multiplicity of infection,

a = allele frequency for one marker,

p = probability of exchange per unit time.

Conclusions. – The effect of genetic drift is certainly not negligible. The evaluation of the effect must await, however, the extension of the treatment to large map distances.

FURTHER EXPERIMENTS ON THE CHEMISTRY OF CROSSING OVER

Sheldon Wolff F. J. de Serres Judith M. Pier

Earlier experiments[12] had shown that the treatment of *Neurospora crassa* at the time of crossing over with the known DNA synthesis inhibitor, 5-fluorodeoxyuridine (FUdR), increased recombination. Treatment with other metabolic inhibitors did not cause such an effect. These experiments were interpreted to indicate that a functional mechanism for DNA synthesis was required when the strands exchanged during crossing over. The present experiments were designed to determine whether FUdR and structurally related pyrimidine nucleosides were consistent in their effect, over a wide range of concentrations, on the frequency of recombination between four closely linked markers. The effect on recombination in each of three regions was determined by treating a cross of two strains of the following genotypes: A + *hist-3* + *nic-2* × a *arg-3* + *ad-3B* +. Crosses were made in 100- by 15-mm petri plates containing filter paper, and aqueous solutions of all chemicals were added 16 hr after protoperithecia were fertilized. This schedule ensured normal fertilization and chemical treatment during the subsequent waves of meiosis in each perithecium.

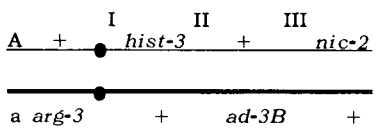
Results. – The results of the experiments are presented in Table 4, in which the percentage increase in recombination in the three regions of the chromosomes to be tested are presented. It may be seen that FUdR at very low concentrations increased recombination in all three adjacent regions of the chromosome. Although previous experiments had shown that low concentrations (10^{-4} *M*) of either uridine or thymidine did not increase recombination, in the present experiments higher concentrations of these compounds were used. The table also shows that at high concentrations both these pyrimidine nucleosides increase recombination by themselves. The mechanism for such an effect is not known, although it is suspected that at these high concentrations feedback inhibitions occur that can inhibit DNA synthesis. The FUdR brings about its inhibition at concentrations far lower than those concentrations of thymidine and uridine that are effective.

Discussion. – The present results confirm those obtained earlier, that is, FUdR, a compound which is known to inhibit the DNA synthesis in bacterial systems, brings about an increase in recombination in *Neurospora*. Such increases have been found in all regions tested so far. A further effect of

[12]Sheldon Wolff, F. J. de Serres, and J. M. Pier, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 32–33.

high concentrations of other pyrimidine nucleosides was also noted. It is not inconceivable that this effect, too, might be the result of an inhibition of DNA synthesis.

Table 4. Effect of Various Inhibitors on Recombination in *Neurospora*



	Increase (%)		
	I	II	III
FUdR 5×10^{-5} / Control	39	61	81
FUdR 5×10^{-6} / Control	79	71	80
FUdR 5×10^{-5} + Urd 5×10^{-3} / Control	60	68	70
FUdR 5×10^{-6} + Urd 5×10^{-4} / Control	73	86	94
FUdR 5×10^{-5} + Thd 5×10^{-3} / Control	78	114	144
FUdR 5×10^{-6} + Thd 5×10^{-4} / Control	41	71	84
Urd 10^{-2} / Control	127	104	166
Urd 5×10^{-3} / Control	64	64	84
Urd 5×10^{-4} / Control	51	46	56
Thd 10^{-2} / Control	41	68	87
Thd 5×10^{-3} / Control	73	82	116
Thd 5×10^{-4} / Control	89	75	117

STUDIES ON THE MECHANISM OF THE EFFECT OF FUdR ON CHROMOSOMES OF *VICIA FABA*

Sandra Bell Sheldon Wolff

Introduction. – It has been shown that 5-fluorouracil deoxyriboside (FUdR) is a specific DNA synthesis inhibitor in virus-infected *Escherichia coli.*[13] In lateral roots of the broad bean, *Vicia faba,* FUdR results in chromosome damage within 3 to 5 hr after the beginning of the FUdR treatment. There is a marked decrease in the mitotic index within 5 hr.[14] Because the time involved in observing chromosome damage is equal to G_2 in untreated roots and also because FUdR is a specific inhibitor of DNA synthesis in *E. coli,* it was originally postulated that the chromosome aberrations in *Vicia* occurred at the S period.[14] It has subsequently been shown that the chromosome damage occurs in G_2 and not in S.[15]

No rejoining of FUdR-induced breaks has been observed. The occurrence of these breaks can be prevented, however, by the addition of exogenous thymidine at concentrations 100 times the concentration of FUdR in the growth medium.[16] Some radiation experiments have been interpreted to mean that FUdR, in concentrations too low to induce chromosome damage, will prevent rejoining of x-ray-induced chromosome breaks. Another interpretation of these experiments is that the x rays are enhancing the FUdR effect.[17] Evidence for this interpretation comes from experiments showing that FUdR treatments are equally effective when given after x rays as they are when given before or during x-ray treatment.[17]

Because it is now known that the effect of FUdR on chromosome breakage occurs in G_2 rather than in S and is then probably independent of the effect of FUdR on DNA synthesis, several experiments were undertaken to see if the mechanism of the effect of FUdR on chromosomes of *Vicia* could be found.

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[13] S. S. Cohen *et al.*, *Proc. Natl. Acad. Sci. U.S.* **44**, 1004 (1958).

[14] J. H. Taylor, W. F. Haut, and J. Tung, *Proc. Natl. Acad. Sci. U.S.* **48**, 190 (1962).

[15] Sandra Bell, Abstract for *Am. Soc. Cell Biol.* (November 1962).

[16] Sandra Bell, Abstract for *Intern. Genetics Congress* (September 1963).

[17] B. A. Kihlman, *Hereditas* **49**, 353 (1963).

Results. – Since a concentration of thymidine equal to the concentration of FUdR did not reverse the chromosome damage, it was decided to see if DNA synthesis was affected in roots grown in equal concentrations of thymidine and FUdR. Roots were grown in a mixture of 10^{-6} *M* FUdR and 10^{-6} *M* H^3-thymidine. Not only was the percentage of aberrant metaphases similar to the percentage of aberrant metaphases in cells without thymidine added to the FUdR, but when autoradiographs were made, the number of grains over the labeled nuclei was found to be the same in the presence of FUdR as in its absence.

To test the hypothesis that FUdR inhibits the rejoining of chromosome breaks, experiments were undertaken to see if FUdR had any effect upon rejoining of x-ray-induced chromosome breaks in G_1 cells of *Vicia*. For the first 30 to 36 hr after the dry seeds of *Vicia* are placed in water, the cells of the root are all in G_1. Seeds were soaked 20 hr in H_2O and were subjected to fractionated doses of x rays separated by 90 min to allow rejoining of the breaks produced by the first dose. Under these conditions the yield of two-break aberrations seen when the cells reach division will be the sum of the yields of the two doses. If FUdR affects rejoining, then by applying it after the first dose, the breaks from this dose will remain open and capable of forming exchanges with the breaks from the second dose. The yields will then approach a yield proportional to the square of the total dose. The results were that the yield of aberrations is not significantly different from the sum of the yield of the two doses when FUdR is supplied during the 90-min period between doses.

Conclusions. – It has been shown that FUdR has two effects. In *E. coli*, and presumably in *Vicia*, there is an effect on DNA synthesis. The chromosome-breaking effect of FUdR can be seen to be independent of the effect on DNA synthesis. Thymidine in concentrations equal to the concentration of FUdR will reverse the first effect, on DNA synthesis, but not the effect on chromosome breakage. The fact that the concentration of thymidine must be higher than the concentration of FUdR to obtain a reversal of the chromosome breakage may mean that there is a competition between FUdR and thymidine for sites within the cell.

The experiments indicating that rejoining of chromosome breaks can occur in G_1 and in the presence of FUdR, and therefore presumably in the absence of DNA synthesis, are consistent with the hypothesis that protein synthesis and not DNA synthesis is necessary for rejoining of chromosome breaks.[18]

CHEMICAL SYNCHRONIZATION OF MERISTEMATIC CELLS IN *VICIA FABA*

Sister Augustine Mattingly

Introduction. – The mitotic inhibitory effect of the pyrimidine analog 5-aminouracil (5-AU) has recently been investigated, and partial synchrony of nuclear stages has been accomplished.[19] This compound acts as a thymine antagonist in bacteria although it is not, *per se*, incorporated into bacterial DNA.[20] Mitotic blockage occurs in onion root tips after a treatment of 5 to 6 hr, and a very high percentage of cells (50–60%) were in mitosis approximately 14 hr after division.[21] Since the advantages of a synchronous system in *Vicia* are obvious, further experiments have been conducted to determine more accurately the schedule of synchrony induced by 5-AU and to determine the physiology of the effect produced.

Results. – Labeling experiments with H^3-thymidine (0.017 mc/cc) indicate that some cells that have completed DNA replication prior to 5-AU treatment (750 ppm in distilled water) can continue through division and into G_1. Cells which are in S, however, appear to be blocked and only proceed to division after the 5-AU is removed. Grain counts of cells labeled and then held for 24 hr in 5-AU show no diminution of label during this time, and the average number of labeled interphases only begins to drop at approximately 8 hr after removal from 5-AU, when the first metaphases begin to be seen. These metaphases are a mixture of labeled and nonlabeled cells. At 14 hr after removal from 5-AU, approximately 40% of the metaphases are labeled. In contrast,

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[18] S. Wolff, *Am. Naturalist* **94**, 85 (1960).

[19] H. H. Smith, C. P. Fussell, and Bertha Kugelman, in press.

[20] A. Wacker *et al.*, *J. Mol. Biol.* **2**, 69 (1960).

[21] P. S. Woods and R. E. Duncan, *Genetics* **37**, 636 (1952).

when cells are treated with 5-AU and subsequently treated with H^3-thymidine for 15 min, the percentage of labeled metaphases 14 hr later rises to about 70%. This indicates that a large population of cells in G_1 at the beginning of 5-AU treatment move into S and are blocked during treatment.

Cells removed from 5-AU are not able immediately to synthesize DNA at full capacity. Roots removed to water after a 24-hr treatment with 5-AU were treated with H^3-thymidine (15 min) at intervals of 0, 1, 3, and 7 hr. Grain counts were made of roots fixed immediately, while mitotic index counts were made on roots held for 14 hr after treatment (Fig. 2). In roots placed in H^3-thymidine immediately, the average grain count is low, and relatively few cells are labeled. In roots placed in H^3-thymidine after a 1-hr recovery, the number of labeled cells increases, although the number of grains per nucleus is still low. After 3 hr recovery, the number of labeled cells is not greatly increased, but the average number of grains per nucleus rises sharply. Finally, after 7 hr, both the average number of labeled cells and the average grain count again decrease.

Preliminary experiments on the physiological basis of the 5-AU effect show that recovery may be enhanced by treatment with certain of the deoxynucleosides (Fig. 3). By far the greatest effect was produced by a mixture of thymidine (750 ppm) and folic acid (75 ppm), although these two chemicals individually did not appear to be stimulatory. Furthermore, while deoxyadenosine and deoxycytosine totally inhibited recovery, deoxyguanosine (750 ppm) apparently stimulated entrance into division, although a high incidence of micro-nuclei in postdivision cells indicates some deleterious effect on cell division itself.

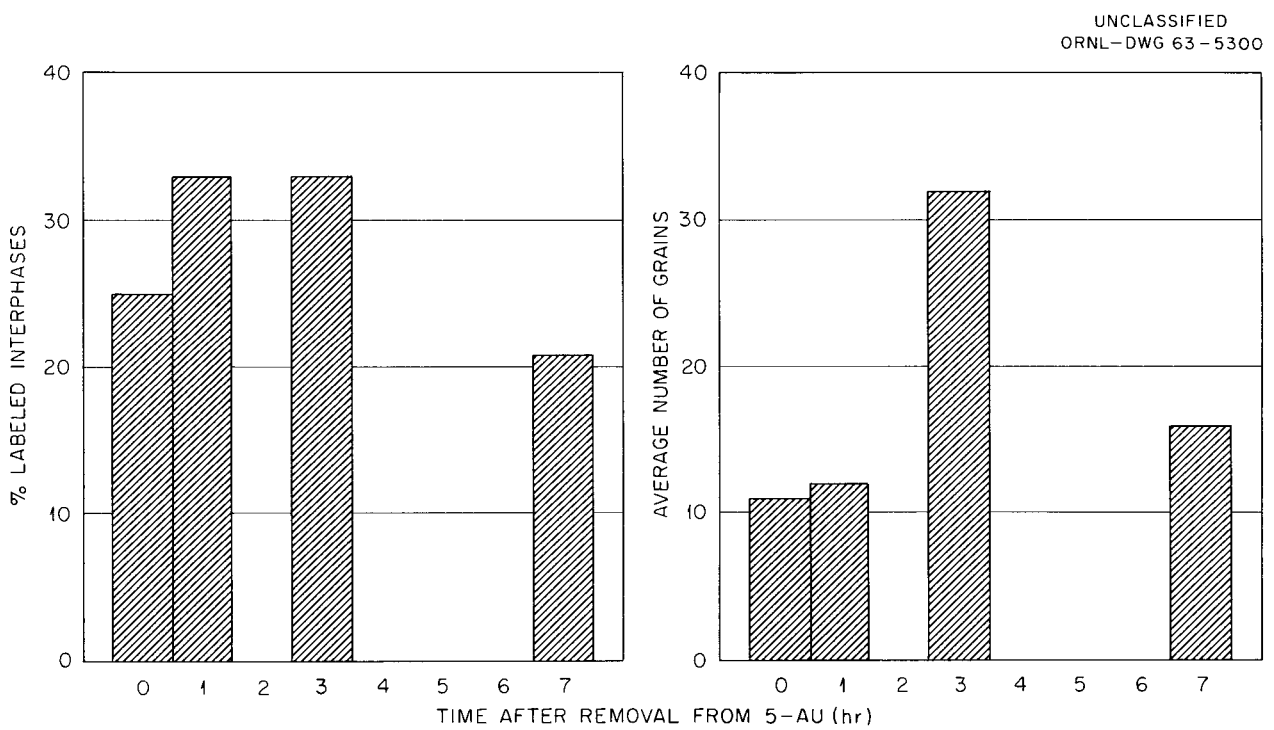

Fig. 2. Comparison of the Percentage of Interphase Cells That Become Labeled with H^3-Thymidine and the Average Number of Grains per Labeled Nucleus at Intervals During the 8 hr Following Removal from 5-AU.

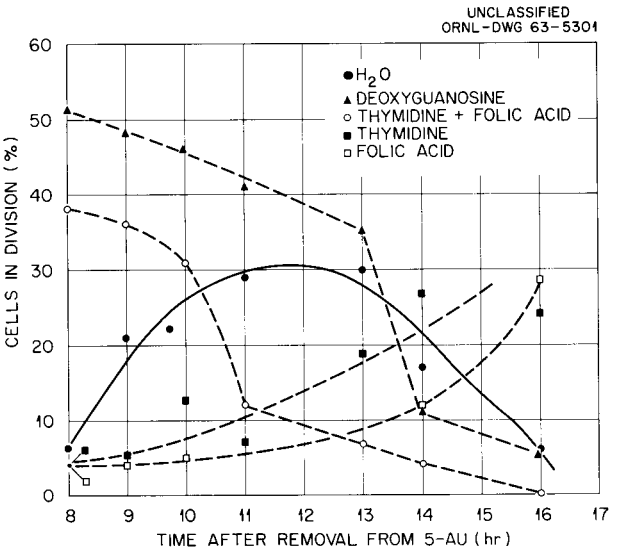

Fig. 3. The Effect of Thymidine and/or Other DNA Precursors on Recovery from 5-AU-Induced Mitotic Delay.

BLOCKAGE OF CELLS IN G_2 BY 5-AMINOURACIL

Sheldon Wolff H. E. Luippold

Introduction. – The pyrimidine analog, 5-aminouracil (5-AU), has been found to induce partial synchrony of *Vicia faba* cells when administered at the concentration of 750 ppm.[22] When actively growing roots of *Vicia* are placed in the compound, the mitotic index slowly falls to zero. If roots are removed from the compound, mitoses again appear, after a delay of approximately 9 hr. A peak of mitosis (60% mitotic index) is observed 14 hr after the roots are removed from the 5-AU. Since this compound has been found to be a thymine antagonist in bacteria, it is thought that it blocks cells in the S period of the cell cycle. However, no critical tests of this hypothesis have been made; and, if the compound is to be used to obtain large numbers of cells in given stages of the mitotic cycle following either its application to or its removal from the roots, then it is imperative that we know exactly where the block occurs so that we might know in what stages of the cell cycle we might expect to find cells. Consequently, root tips of *V. faba* were labeled with H^3-thymidine at a concentration of 4 μc/ml for either 15 min (flash labeling) or for 1 hr. The roots were then placed in 750 ppm of 5-AU; at various times after this the roots were sampled, and the mitotic index of both total cells and labeled cells was obtained.

Results. – The results of the first experiment on flash-labeled cells showed that the mitotic index fell between 2 to 9 hr in the 5-AU-treated material. Labeled cells appeared at metaphase in the water controls but did not appear in those cells treated with 5-AU. This indicated that the block might be all through S, so that cells that were in S would not proceed to metaphase.

In an attempt to be more critical, studies were made with roots that had been labeled for 1 hr. Under these conditions all the cells in S would be labeled, and the cells that were at the end of S at the time the label was first applied would have moved for 1 hr into G_2. The results are shown in Table 5. It may be seen that the mitotic index did not fall between 5 to 9 hr in water and that a large proportion of the cells in division were labeled cells either from S or the first hour of G_2. When the roots were placed in 5-AU, however, the mitotic index fell over this period of time. Only a few labeled cells appeared at metaphase at 5 and 6 hr. No labeled cells appeared between 7 to 9 hr.

Table 5. Effect of Treatment with 750 ppm 5-AU on Mitotic Index[a]

Time (hr)	Mitotic Index			
	Water Controls		5-AU	
	Total	Labeled	Total	Labeled
5	7.9	4.084	8.15	0.187
6	14.0	8.13	4.18	0.185
7	13.4	9.39	6.01	0.0
8	15.4	8.67	2.60	0.0
9	14.7	10.14	3.9	0.0

[a] Cells were labeled for 1 hr before 5-AU treatment.

[22] H. H. Smith, C. P. Fussell, and Bertha Kugelman, in press.

Discussion. – The results of the present experiment clearly show that 5-AU blocks cells not only in S but also in G_2 of the mitotic cycle and that after treatment with 5-AU, one can expect to find some cells from early G_2 in the roots. This is consistent with the results from an earlier radiation experiment (Wolff and Luippold, unpublished data), in which it was found that after treatment with 5-AU, if flash-labeled roots were irradiated with 100 r of x rays, unlabeled chromatid aberrations were present when the cells reached metaphase. These unlabeled chromatid aberrations evidently came from the unlabeled G_2 cells.

EFFECT OF FAR RED LIGHT ON CHROMATID ABERRATIONS INDUCED BY X IRRADIATION OF *TRADESCANTIA* MICROSPORES

T. H. Ma Sheldon Wolff

Introduction. – Withrow and Moh[23,24] reported that far red (710–820 mμ) radiant energy increases the yield of chromatid aberrations induced by x rays in *Vicia faba*. Wolff and Luippold[25] studied the chromatid aberrations induced throughout the G_2 period of the mitotic cycle. Their results indicated that a peak of aberrations occurred about 15 hr after 150 r of x rays and about 18 hr after 150 r of x rays, plus 3 hr of far red treatment. The increase of aberrations reported by Withrow and Moh in their material fixed 24 hr after x irradiation consequently appeared to be caused by a delay of the mitotic cycle.

The present investigation intends to ascertain whether far red light increases by the same mechanism the chromatid aberrations induced in *Tradescantia*.

Results. – Young inflorescences with a portion of stem attached of *Tradescantia paludosa* (Sax clone 3) were excised and maintained in aerated spring water for about 7 to 10 hr prior to treatment. One group was pretreated with far red light for 3 hr and x irradiated afterward. A second group was x irradiated first and then treated with far red light. A third group served as a light control and was x irradiated and kept in light after treatment. A fourth group served as a dark control and was x irradiated and kept in the dark for 3 hr. An exposure of 100 r of x rays and 3 hr of far red (760 mμ) light was used. Material was fixed at $1\frac{1}{2}$-hr intervals, starting from 12 hr after x irradiation up to 21 hr. Newcomer's fixative and the acetocarmine squash method were used to prepare the slides. They were scored for chromatid aberrations. Both control groups showed a high peak of aberrations at 15 hr after x irradiation. Far red pretreatment and posttreatment groups showed two peaks of aberrations. With pretreatment, the first peak appeared at 12 hr, and the second at $16\frac{1}{2}$ hr. With posttreatment, the first peak appeared at $13\frac{1}{2}$ hr, and the second at 18 hr.

Discussion. – In *Tradescantia* microspores the far red light (760 mμ) given either before or after x rays is able to alter the time course of the appearance of aberrations induced during the G_2 period of the mitotic cycle. Far red light seems to delay mitosis and so shift the appearance of the peak yields of aberrations to later times. The peak prior to the regular peak reveals another sensitive stage at late G_2 that is not ordinarily seen because of the radiation-induced pycnosis found in cells reaching metaphase within the first few hours after radiation.

DNA SYNTHESIS RATE IN ONION ROOT MERISTEMS DURING X IRRADIATION

Sheldon Wolff R. W. Oliver

Introduction. – Das and Alfert[26] reported in 1961 that low doses of x rays stimulate DNA synthesis in onion root tip meristems. Since this is the only

[23] R. B. Withrow and C. C. Moh, *Radiation Res.* **6**, 491 (1957).

[24] C. C. Moh and R. B. Withrow, *ibid.* **11**, 18 (1959).

[25] S. Wolff and H. E. Luippold, "Progress of Photobiology," in *Proc. 3rd. Intern. Congr. Photobiol.*, pp 457–60, 1960.

[26] Nirmal K. Das and Max Alfert, *Proc. Natl. Acad. Sci. U.S.* **47**, 1 (1961).

report in which an increase in synthesis was noted following radiation and since the doses used (~800 r) should not directly affect enzyme systems in the living cell, an attempt was made to confirm their experiments and to gain some insight into the mechanisms involved.

Onion roots were grown from seed onions in vials of distilled water. When the roots were $1\frac{1}{2}$ to 2 in. long, they were placed in H^3-thymidine (16.7 μc/ml) and irradiated with 800 r at 50 r/min. Immediately after irradiation half of the roots were picked from all onions and fixed. The onions were then replaced in the H^3-thymidine for another 24 min, at which time the remaining roots were picked. Feulgen squashes were made of the root tip material, and autoradiographs were prepared. Grain counts were made of the slides.

Results. – The results show (Table 6) that if roots that were $1\frac{1}{2}$ to 2 in. long were irradiated with 800 r, the average number of grains increased in the irradiated material when it was scored either immediately after irradiation (16 min) or 24 min after irradiation (40 min). This was true even in cases in which the nonirradiated roots were placed in irradiated medium. The latter was done to see whether or not the possible effect came about because irradiated medium made roots more permeable to thymidine so that more would get into the root and thus be incorporated.

It was noted in the slides that only a very low percentage of the cells were in mitosis and that a low percentage of the cells were labeled, indicating that when the roots from seed onions were $1\frac{1}{2}$ to 2 in. long, they were not growing actively. When the experiment was repeated with short roots, it was found that the stimulatory effect of radiation disappeared. In these short roots the mitotic index was twice that in the long roots, and the percentage of cells labeled was higher.

Discussion. – The data obtained indicate that, if the experimental procedure of Das and Alfert is followed exactly, the stimulation of the DNA synthesis is observed. This increased production of DNA, however, seems to be a function of root length. The stimulatory effect is found only in long semimoribund roots that are not growing actively. Normal active roots do not show this stimulatory effect. The reasons as to why x rays can induce a stimulation of DNA synthesis in inactive roots are as yet obscure.

TECHNIQUE FOR CULTURING *TRADESCANTIA* MICROSPORES

J. D. Caponetti Sheldon Wolff

Introduction. – In the past, only little has been done with the problem of culturing living cells from anthers. Shimakura[27] cultured microsporo-

[27]K. Shimakura, *Cytologia* **5**, 363 (1934).

Table 6. Incorporation of H^3-Thymidine into Roots of Onion

Type of Root	Time of Fixation (min)	800 r			0 r		
		Av No. of Grains	Mitotic Index	Cells Labeled (%)	Av No. of Grains	Mitotic Index	Cells Labeled (%)
Long	16	22.4	2.4		14.6	2.5	
	40	36.5	1.7		21.7	1.1	
	16	21.4	2.6	17.2	11.5[a]	2.1[a]	12.3[a]
	40	42.9	1.4	14.1	21.6[a]	1.5[a]	14.9[a]
Short	16	10.8	4.0	22.5	10.3	3.8	24.6
	40	28.0	4.5	43.5	28.9	3.8	33.1

[a]These roots were placed in H^3-thymidine and irradiated with 800 r.

cytes of *Tradescantia virginiana* L. by the hanging-drop method. Although the cells plasmolyzed slightly and the chromosomes swelled, undamaged cells proceeded through meiosis. Shimakura also reports[28] that the microsporocytes of *Trillium kamtschaticum* can be cultured by the same method. In this case, however, the cells proceed only to anaphase I. Recently, Walker,[29] using slight alterations of a basic medium described by Taylor,[30] cultured cells from anthers of *Tradescantia* spp. in various stages of development by the liquid-paraffin hanging-drop method.

The culture of isolated anthral cells would indeed be very useful in research on chromosomal aberrations. Such isolated cells in known stages of division could be directly exposed to various types and doses of irradiation and/or chemical compounds, and chromosomal aberrations could be readily studied at metaphase. Some irradiation studies have already been done on pollen grains, as described in a recent review;[31] but as yet no work has been done with isolated cells from the earlier stages of microsporogenesis.

The object of the present study was to devise a technique for culturing anthral cells so that the various stages of division could be clearly recognizable under the light microscope. To date, only the microspore stage and its subsequent mitotic stages have been studied.

The plant used was *Tradescantia paludosa* Sax Clone 3, since this clone is known to have very few spontaneous chromosomal aberrations and has been used extensively in chromosomal studies.

Results. – Clearly recognizable mitotic stages in the microspore may be obtained by utilizing the following technique. An anther at the appropriate stage is gently crushed with a small scalpel onto microslides that have been subbed with fresh egg albumin. The slides have two hemispherical depressions. The anthral walls are removed, and the cells are gently spread over the surface. A $^7/_8$-in. square cover glass is placed evenly over the surface, and the slide is quickly placed on the stage of a microscope which is equipped with phase optics. Sterile nutrient medium is allowed to drop at a regulated pace onto the depression on the right-hand side of the slide from a specially constructed burette. The medium is removed by means of a capillary tube from the depression on the left-hand side of the slide.

Discussion. – The described technique operates efficiently for long periods of time. It also satisfies several requirements of free cell culture. The constant flow of medium through the culture maintains the cells uncontaminated by microorganisms, well-oxygenated, and supplied with known quantities of nutrients. Removal of the medium from the other end of the slide maintains the cells uncontaminated and removes waste products of cellular metabolism. At any time the cells may be treated as desired, and the effects may be observed and photographed.

Although the culture technique is successful, the correct nutrient medium persists as a problem. The various kinds of media which are regularly used for the culture of plant tissues have failed either to induce mitosis in the microspore nucleus or to allow the conclusion of mitosis when cells in the midst of division are placed on the slide. The cells and their vacuoles at first swell and later shrink and appear to plasmolyze. For those cells that are in a particular stage of mitosis, the chromosomes become shorter and thicker than they were and appear to clump and fuse together. This is true for this clone of *Tradescantia* even when treated with Walker's medium, either plain or supplemented with 15% coconut water. The pH seems to be critical. Sterilizing the medium by autoclaving changes the pH. Therefore, media must be sterilized by millipore filtration. The pH of anthral contents at the microspore stage is 5.5, as determined by crushing an anther on p-Hydrion test paper. All media, therefore, have been adjusted to pH 5.5 with potassium hydroxide. But still the media have failed; this and many other factors have to be solved before a workable medium is found.

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[28] K. Shimakura, *Cytologia*, Fujii Jub. Vol., 256 (1937).

[29] G. W. R. Walker, *Proc. Genetics Soc. Canada* 2, 18 (1957).

[30] J. H. Taylor, *Am. J. Botany* 37, 137 (1950).

[31] J. L. Brewbaker and G. C. Emery, *Radiation Botany* 1, 101 (1962).

MUTAGEN SPECIFICITY IN *NEUROSPORA CRASSA*

F. J. de Serres W. E. Barnett
H. E. Brockman H. G. Kølmark

Do different mutagenic agents produce a different spectrum of induced mutation? On the currently accepted theory of information transfer, a definite correlation might be expected between the type of genetic alteration in a given locus and the type of altered polypeptide chain specified. Mutagenic treatments that produce such gross alterations in the DNA as deletion of a large sequence of nucleotides should result in a grossly altered, or no polypeptide chain; whereas treatments that effect a single base pair substitution could result in single amino acid substitutions. Such differences in the type of polypeptide chain specified by these different types of genetic alterations can be detected by a variety of biochemical and genetic tests. Furthermore, if different physical and chemical mutagens produce predominantly base pair deletion or base pair substitution, then the percentages of mutants with slightly modified or with grossly altered enzymes should vary in samples of different mutagenic origin.

To investigate the problem of mutagen specificity in *Neurospora*, we have compared the spectrum of mutation in the *ad-3* region produced by a variety of different agents in tests that can distinguish between the two types of mutant enzyme: (1) allelic complementation and (2) leakiness (resulting from an incomplete block in the purine biosynthetic pathway).

The comparison involves *ad-3A* or *ad-3B* mutants occurring spontaneously or induced in wild type 74A or isogenic derivatives. In contrast to such studies present in an earlier report,[32] all mutants were recovered in forward-mutation experiments using the direct method under comparable conditions.

Results. – Aliquots of a conidial suspension of each mutant were adjusted to 0.75×10^5/ml of medium, and 0.75-ml aliquots were tested in pairwise combination with 18 different tester strains that define the *ad-3A* cistron and 17 complons with the *ad-3B* cistron and an additional *hist-3* tester as a control (to detect the presence of incompatibility factor mutations). Such heterokaryon tests determine the place the *ad-3* mutation occurred (at the *ad-3A* or *ad-3B* locus) and the complementation patterns of the *ad-3B* mutants on the complementation map of the *ad-3B* cistron. In addition, each mutant is tested alone, and any detectable growth here is an indication of an incomplete block or leakiness. The results of such tests on 788 mutants occurring spontaneously or induced by various physical or chemical agents are summarized in Table 7. A comparison of the complementation patterns of the *ad-3B* mutants is incomplete and will be given in a subsequent report.

Discussion. – The data show marked differences in the percentage of leaky mutants at each locus as a function of mutagenic origin. Furthermore, the highest percentages were found among the samples induced by HNO_2 and 2-aminopurine (2-AP), mutagens believed to produce mutation predominantly by base pair substitution events. The lower percentages of leaky mutants are found after treatment with those agents known or suspected to give rise to mutations by mechanisms other than base pair substitution. The same trend was found with allelic complementation among the *ad-3B* mutants. It is of particular interest that the percentages of leaky and complementing mutants in the 2-AP and HNO_2 samples are in excellent agreement. Both ultraviolet (uv) and ethyl methane sulfonate (EMS) induce mutations by both base pair substitution and deletion events, and lower percentages were found with both of these mutagens than with 2-AP or HNO_2. The mechanisms by which meso-diepoxybutane (DEB) produces mutations are unknown; however, in a broad spectrum experiment on Penicillium[33] no difference was found on the relative induction of morphological or biochemical mutants with either DEB or uv. It is of interest that in the present experiments these two agents also give comparable data. The mechanisms of mutation produced by each of these agents can be determined by testing samples of mutants for their reversibility with specific agents. Such tests have been attempted

[32] F. J. de Serres, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1961*, ORNL-3201, p 40.

[33] M. Westergaard, *Abhandl. Deut. Akad. Wiss. Berlin, Kl. Med.* 1960, 116.

Table 7. Correlations Between the Mutagenic Origin of *ad-3* Mutations and the Percentage of Leakiness and Percentage Allelic Complementation

Mutagenic Treatment	Total *ad-3* Mutants	*ad-3A*		*ad-3B*		
		Total	Percentage Leaky	Total	Percentage Leaky	Percentage Complementing
Spontaneous	30	11	18.2	19	5.3	36.8
Radiation						
X ray	140	47	6.4	93	3.2	33.6
Uv	164	53	11.3	111	14.4	45.0
Chemicals						
2-AP	46	11	45.4	35	22.9	74.3
HNO_2	144	33	42.4	111	23.2	73.2
EMS	126	32	21.8	94	8.5	53.2
DEB	138	38	13.2	100	10.0	53.0

on HNO_2- and EMS-induced mutants, as discussed in a previous report.[34] In these tests it was possible to determine that these mutagens do produce base pair substitution events in *Neurospora* and that in these cases many of the mutations were transition events.

The data from the present and previous experiments indicate that each of these mutagenic agents produced a characteristic spectrum of mutation in *Neurospora*. The difference in the spectrum of mutation can be readily demonstrated with a variety of genetic tests.

A CORRELATION BETWEEN X-RAY DOSE AND THE REPARABILITY OF X-RAY-INDUCED *ad-3* MUTANTS OF *NEUROSPORA CRASSA*

B. B. Webber F. J. de Serres

The use of a balanced heterokaryon in forward-mutation studies with x rays on *Neurospora crassa*[35] has permitted the recovery of two different classes of purple, adenine-requiring mutants altered in the *ad-3* region. These include (1) *reparable mutants* ($ad\text{-}3^R$) that are capable of growth as homokaryons on adenine-supplemented medium, and (2) *irreparable mutants* ($ad\text{-}3^{IR}$) that are incapable of growth as homokaryons even on completely supplemented medium. The genetic alterations resulting in $ad\text{-}3^{IR}$ mutants are not known, but evidence from similar experiments with HNO_2 treatment[36] suggest that $ad\text{-}3^{IR}$ mutants must result from extragenic alterations, and genetic analysis[37] by homology tests of $ad\text{-}3^{IR}$ mutants suggests that they are the result of chromosome deletion.

If $ad\text{-}3^R$ mutants are the result of single-hit events in the *ad-3A* or *ad-3B* locus and $ad\text{-}3^{IR}$ mutants result from two-hit events, then the relative proportions of these two classes of mutants should vary markedly as a function of x-ray dose. To provide information about the kinetics of *ad-3* mutant induction and to make possible genetic analysis of the spectrum of induced mutation at each dose, a series of experiments were performed with x-ray exposures of 1, 2, 5, 10, 20, and 40 kr. The dose-survival curve was exponential, suggesting a single-target mode of cellular inactivation,[38] and the frequency of *ad-3* mutants among the survivors increases as the 1.3 power of the dose.

[34] W. E. Barnett, H. E. Brockman, and F. J. de Serres, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p 45.

[35] F. J. de Serres and Rosalie S. Osterbind, *Genetics* 47, 793 (1962).

[36] H. E. Brockman, F. J. de Serres, and W. E. Barnett, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 36–37.

[37] F. J. de Serres, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 34–36.

[38] B. B. Webber and F. J. de Serres, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 37–38.

Results. – The genetic classification of mutants from doses of 1 through 10 kr is presented in Table 8. It is apparent that at low x-ray exposures (1 and 2 kr) the majority of the *ad-3* mutants are reparable but that the fraction of *ad-3* mutants which are irreparable increases markedly with increasing dose. The absolute frequency of *ad-3*^{R} mutants per 10^6 survivors increases approximately linearly with dose, while *ad-3*^{IR} mutants follow more nearly the square of the dose. Furthermore, *ad-3A ad-3B* double mutants (always irreparable) are infrequent at low doses.

Discussion. – The x-ray dose-response data can be explained by the hypothesis that *ad-3*^{R} mutants occur as a result of alterations caused by the action of a single ionizing event, while *ad-3*^{IR} mutants are caused by the cooperative action of two (or more) independent alterations, neither of which by itself induces an *ad-3* mutant. On the simplest interpretation, these alterations occur in different regions, predominantly between or outside the *ad-3A* and *ad-3B* loci. They interact to produce a functional deletion of the intervening genetic material, including one or both of the *ad-3* loci.

It has been observed[36] that HNO_2 induces neither *ad-3*^{IR} mutants nor *ad-3A ad-3B* double mutants. The difference between mutagenic effects of x rays and HNO_2 might best be attributed to a difference between types (rather than distribution) of genetic alteration produced. If HNO_2 produces almost exclusively single base pair substitutions, as is generally supposed, then it is not surprising that two such alterations would fail to inactivate the genetic region separating them.

Additional experiments extending the observations of mutation induction and including samples of mutants derived from conidia exposed to 20 or 40 kr of x rays are in progress.

I.C.R. NO. 170–INDUCED *ad-3* MUTATION IN *NEUROSPORA CRASSA*

H. E. Brockman

The compound 2-methoxy-6-chloro-9-(3[ethyl-2-chloroethyl]aminopropylamino) acridine dihydrochloride (I.C.R. No. 170) contains an acridine ring and a monofunctional alkylating agent (also called a "one-armed" nitrogen mustard group).

Table 8. Distribution of Types of *ad-3* Mutants as a Function of X-Ray Exposure

Factors: 250 kvp; 30 ma; 3-mm aluminum filter; 1 kr/min

X-Ray Exposure (kr)	Survival (%)	Class	Number of *ad-3* Mutants Classified						Percentage at Given Dose	Frequency (per 10^6 Survivors)
			ad-3A	*ad-3B*	*ad-3A ad-3B*	*ad-3A ad-3B nic-2*	*ad-3B nic-2*	Total		
0	100	*ad-3*^{R}	1	2	0	0	0	3		
		ad-3^{IR}	0	0	0	0	0	0		
1	93.5	*ad-3*^{R}	27	67	0	0	0	94	95	4.7
		ad-3^{IR}	2	1	2	0	0	5	5	0.2
2	83.5	*ad-3*^{R}	24	68	0	0	0	92	93	10.5
		ad-3^{IR}	3	2	2	0	0	7	7	0.8
5	61.5	*ad-3*^{R}	41	58	0	0	0	99	87	33.5
		ad-3^{IR}	4	8	2	1	0	15	13	5.0
10	50.5	*ad-3*^{R}	20	53	0	0	0	73	62	62.5
		ad-3^{IR}	11	17	12	3	1	44	38	38.3

It is a potent antitumor agent in mice[39] and a strong mutagen in *Drosophila*.[40] Preliminary experiments have shown that it is also strongly mutagenic in *Neurospora*.

Suspensions of conidia in water were treated with I.C.R. No. 170. All operations were performed under red light to prevent photodynamic interactivation and mutation induction. The spontaneous frequency of *ad-3* mutation is approximately 0.3×10^{-6}. An 8-hr exposure to 1 and 5 μg of I.C.R. No. 170 per milliliter increased the frequency among the survivors to 143×10^{-6} and 1629×10^{-6} respectively.

The mutagenic activity of this compound is probably not due to the acridine ring, because the acridines are not mutagenic in *Neurospora* in the absence of visible light.[41] It is likely, however, that the acridine ring might increase the mutagenic effectiveness of the one-armed nitrogen mustard because acridines bind strongly to DNA.[42]

[39]H. J. Creech *et al.*, *Cancer Res.* **20**, 471 (1960).

[40]E. A. Carlson and I. I. Oster, *Genetics* **47**, 561 (1962).

[41]H. E. Brockman, unpublished results.

[42]R. F. Beers *et al.*, *Nature* **182**, 242 (1958).

[43]E. H. Y. Chu, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 38–40.

ULTRAVIOLET ACTION SPECTRA FOR CHROMOSOME ABERRATIONS OF THYMIDINE ANALOG-SUBSTITUTED MAMMALIAN CELLS *IN VITRO*

E. H. Y. Chu

Introduction. – There is extensive evidence that the incorporation of halogenated thymidine analogs into the DNA of bacteriophages, bacteria, and mammalian cells greatly enhances their radiosensitivity. We have studied[43] the action spectra for uv-induced chromosome aberrations in Chinese hamster cells *in vitro* which had been pretreated with 5-bromodeoxyuridine (BUdR) or 5-iododeoxyuridine (IUdR). These studies have now been completed, permitting certain conclusions on the molecular basis for the uv-induced chromosome aberrations.

Materials and Methods. – Experimental procedures were the same as previously described.[43] The data presented here include only those experiments in which 10^{-5} *M* of either analog was included in the culture medium for 48 hr prior to irradiation. Ultraviolet surface exposure of 100 ergs/mm^2 was given for each wavelength series.

Results and Conclusions. – The data are plotted in Fig. 4. Since the results are reproducible in several repeat experiments (over 10,000 cells analyzed), they are pooled. The action spectrum for analog-unsubstituted (TdR) cells is approximately parallel to the uv absorption spectrum of nucleic acids. The analog-substituted cells exhibit a marked increase in radiosensitivity when exposed to uv. The action spectra for BUdR-labeled and IUdR-labeled cells are also similar to the uv absorption spectra of these analogs.[44] Our results support the view that the DNA molecule is the primary target of the uv injury which

[44]A. Wacker in *Progress in Nucleic Acid Research* (ed. by J. N. Davidson and W. E. Cohn), vol 1, pp 369–99, Academic Press, New York, 1963.

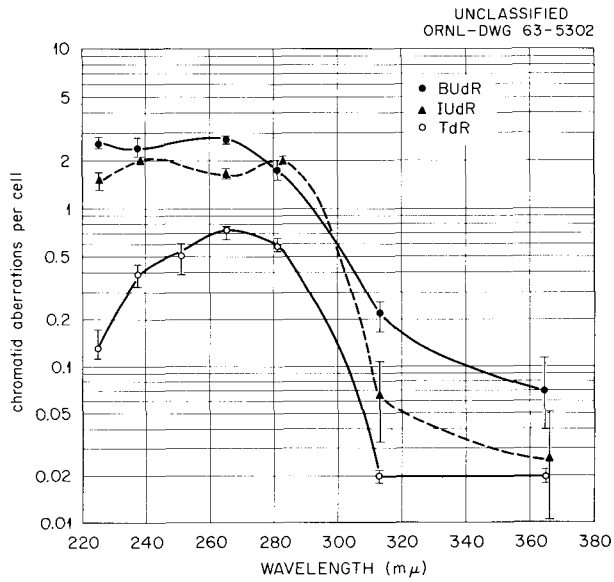

Fig. 4. Relative Effectiveness in the Induction of Chromosome Aberrations by Ultraviolet at Various Wavelengths in Chinese Hamster Cells With or Without Incorporation of Halogenated Thymidine Analogs. Confidence limits at 95% level are indicated.

leads to chromosome aberrations. The modification of the radiosensitivity of this molecule *in situ* by halogenation could depend either on intrinsic increase in radiation lability or on partial or complete loss of the capacity of the DNA molecules to undergo the process of enzymatic or nonenzymatic repair.[45] Experiments designed to answer some of these questions in relation to chromosome aberrations and to determine the relative radiosensitivity of the nucleic acid base pairs to uv and x rays are in progress.

TRIPLOID-DIPLOID CHIMERISM IN A MALE TORTOISE-SHELL CAT

E. H. Y. Chu

Introduction. – The black and yellow coat colors in cat have been thought to be controlled by a pair of allelic, codominant sex-linked genes. When homozygous or hemizygous, the coat has one or the other color; but in the heterozygous state a mixed coat color results which is commonly known as tortoise-shell. On rare occasions, however, male cats with the same phenotype have been reported; most but not all of these have been proven sterile. A number of theories have been put forward to account for its origin, but none of them has received universal support.

Results. – We recently studied the somatic chromosomes of another tortoise-shell tomcat by tissue culture techniques. The cat was sterile. Spermatogenesis was abnormal, with occasional presence of the type A spermatogonia. A mixture of triploid (57 chromosomes including XXY, Fig. 5) and normal female (38, XX) cells were found in cultures derived from the ear and the peritoneum, indicating the pre-existence of the chimeric condition in the cat.

Discussion. – Several mechanisms for the origin of this particular chimera are suggested, including suppression of second polar body, double fertilization, and fusion of zygotes.

It is postulated that the sex-linked allelic genes for the black and yellow coat colors in cat follow the simple Mendelian inheritance and that the tortoise-shell phenotype is due to genetic inactivation of either X chromosome in a heterozygous female. It is further suggested that a male tortoise-shell cat basically possesses an XXY sex chromosome constitution and is heterozygous for the sex-linked coat-color genes, although other types of sex chromosome anomalies and mosaicism can give rise to the same phenotype in males.

AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF MOUSE HEMOGLOBINS

R. A. Popp Diana M. Popp Brenda C. Webb

Introduction. – The primary structure of the α- and β-chains of mouse hemoglobins are controlled by two loci, *Sol* and *Hb*, which segregate independently.[46] Several strains of mice possessing alleles at *Hb* and *Sol* have been identified.[47] Variations on paper chromatograms of tryptic peptides of several hemoglobin phenotypes presumably represent amino acid substitutions. Thus, the amino acid composition of the tryptic peptides of the α-chains of mouse hemoglobins is presently being studied.

Methods. – The α-chain polypeptide is digested by 0.01% trypsin, which selectively splits arginyl and lysyl bonds. The resulting polypeptide fragments (tryptic peptides) are being separated by column chromatography. Partial separation is obtained on Dowex 50-X2 resin, and each fraction is further purified by secondary chromatography on Dowex 1-X2 (ref 48). The tryptic peptides are hydrolyzed by 6 *N* HCl, and the amino acids released are quantitatively determined by a Spinco amino acid analyzer.

Results and Discussion. – The amino acid analyses of the tryptic peptides are given in Table 9. An amino acid difference for the number of

[45] W. Szybalski in *Research in Radiotherapy* (ed. by R. F. Kallman), pp 162–80, Natl. Acad. Sci.–Natl. Research Council, Washington, D.C., 1961.

[46] R. A. Popp, *J. Heredity* **53**, 77–80, 142–151 (1962).

[47] R. A. Popp, *Science* **140**, 893 (1963).

[48] W. A. Schroeder *et al.*, *Anal. Chem.* **34**, 1570 (1962).

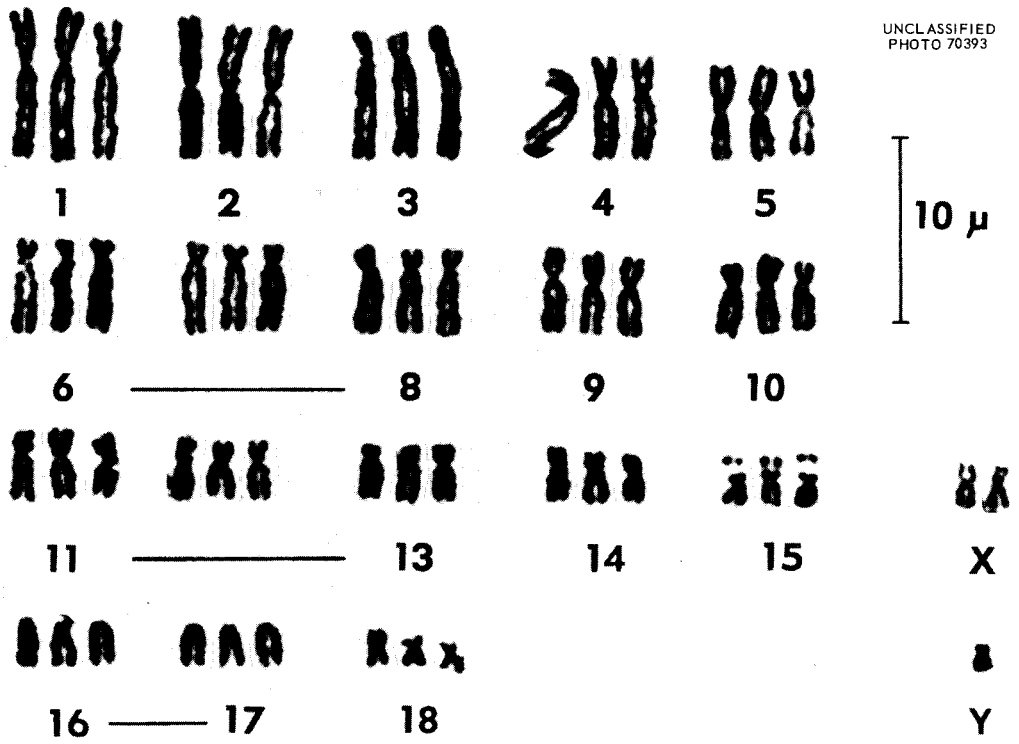

Fig. 5. Karyotype of a Triploid Cell Present in the Chimeric Male Tortoise-Shell Cat.

Table 9. Amino Acid Composition of Tryptic Peptides from the α-Chain of C57BL Hemoglobin

Peptide No.	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Leu	Tyr	Phe	Try	Ileu	Cys
1	1			1		1	1		1		1		1					
2	1			1		1											1	
3	1								1	2						1		
4		1	1				3		4	3			1	1			1	
5	1				2	1		1		1		1			2			
6	1	2		1	1	2	1	1	1	1	2			1	2			
7	1	1							2									
8	1																	
9[a]	1	3		5		2		1	2	8	1		6					
10			1										1					
11	1			2				1			2				1			
12																		
13																		
14			1										1					

[a]Amino acid substitution, Asp → Thr, found in this peptide of strain BALB/c hemoglobin.

aspartic acid and threonine units in peptide αT-9 of C57BL and BALB/c hemoglobins was observed. The position of the substitution is not known. Furthermore, the insoluble core peptides, αT-12 and -13, remain to be analyzed. Tentatively, we conclude that the solubility differences associated with Sol^1 of C57BL and Sol^2 of BALB/c hemoglobins are reflections of changes in the composition of amino acids in αT-9.

REGRESSION OF (C57BL × 101)F_2 MARROW GRAFTS IN LETHALLY IRRADIATED (C57BL × 101)F_1 RECIPIENTS

R. A. Popp Diana M. Popp

Introduction. – Bone marrow from parental strain mice infused into lethally irradiated F_1 hybrids usually establishes long-term hematopoietic grafts; strain C57BL marrow is an exception.[49] Popp *et al.*[50] showed that C57BL marrow repopulates lethally irradiated F_1 recipients less efficiently than marrows of several other strains of mice. This study was done to determine whether factors affecting the abilities of C57BL and 101 marrow to provide long-term grafts in irradiated F_1 recipients segregate as simple Mendelian traits.

Methods. – (C57BL × 101)F_2 mice were produced from (C57BL × 101)F_1/Cum mice. Each mouse was classified for sex, coat color, hemotype, and serotype. The bone marrow cells of one F_2 donor were injected intravenously into four lethally irradiated F_1 recipients (approximately 10×10^6 marrow cells per recipient). Marrow of 162 F_2 donors was injected into 648 F_1 recipients. Each F_1 recipient was tested for the presence or disappearance of donor-type erythrocytes at 60, 120, and 180 days after irradiation.

Results and Discussion. – Marrows from 12.7% of the F_2 donors failed to establish long-term grafts in irradiated F_1 recipients. Thus, the factors associated with or that mimic the reduced ability of C57BL marrow to establish long-term grafts in F_1 recipients is inheritable, but the nature of these factors is not known. The results in Table 10 show that the factors are not sex-linked and they segregate independently from *Hb*, *A*, and *H-2*. Therefore, factors that are associated with the regression of C57BL marrow grafts may be different from the *H-2*-associated differences in initial growth noted by Cudkowicz and Stimpfling.[51] Collaborative experiments are planned to determine this possibility.

[49] R. A. Popp, *J. Natl. Cancer Inst.* **26**, 629 (1961).

[50] R. A. Popp, G. E. Cosgrove, and D. M. Popp, *Ann. N.Y. Acad. Sci.*, in press.

[51] G. Cudkowicz and J. H. Stimpfling, *Genetics* **48**, 886 (abstr.); manuscript in preparation.

Table 10. Frequency of Regression of Grafted F_2 Marrow of Designated Phenotypes

Phenotype		Number of Mice	Number of Regressions	Frequency of Regressions (%)
Hb^1Hb^1		73	7	9.6
Hb^2Hb^2		85	13	15.3
A-		113	15	13.3
aa		45	5	11.1
H-2 b/b		60	6	10.0
H-2 b/k		58	12	20.7
H-2 k/k		40	2	5.0
♀ ♀		73	8	11.0
♂ ♂		85	12	14.1
Donor Sex	Recipient Sex			
♀	♀	46	5	10.9
♂	♂	33	4	12.1
♀	♂	27	5	18.5
♂	♀	52	6	11.5

Drosophila Cytology and Genetics

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Radiation Genetics

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Chromosomal Behavior and Biochemical Genetics

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Rhoda F. Grell
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Pseudoallelism and Immunogenetics

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[a]Leave of absence, University of Washington, Seattle, June–September 1963.
[b]USPHS Fellow.
[c]Visiting investigator from abroad.
[d]Loanee.
[e]Research associate.
[f]Student trainee.
[g]Leave of absence, University of Chile, Santiago, February 1963–February 1964.
[h]Consultant.

CONSTRUCTION AND PRELIMINARY INVESTIGATION OF THE BEHAVIOR OF A TANDEM RING COMPOUND X CHROMOSOME

L. Sandler[1] D. L. Lindsley

A tandem ring compound X chromosome (TRX) has been synthesized according to the scheme outlined in Fig. 6. To accomplish step A, 30 baskets of matings containing 10 females and 10 males per bottle of the genotype indicated were used. No recombinants were recovered. The experiment was then repeated, but the parental females were treated with 2 kr of x rays. Seven tandem metacentric compound X chromosomes that were still heterozygous for the parental markers were recovered from the second run. Stocks were produced from these seven compound-bearing females; TMX-bearing females were mated in large numbers, and their progenies were examined for matroclinous daughters that had lost y^+ and B^S by the recombinational event B. Several TRX chromosomes were recovered in this way, and preliminary counts have been made on one of them. Nearly all the TRX chromosomes that have been recovered to date have been derived from the same TMX chromosome. The reason for the different behavior of the independently derived TMX chromosomes is not understood.

[1]Department of Genetics, University of Washington.

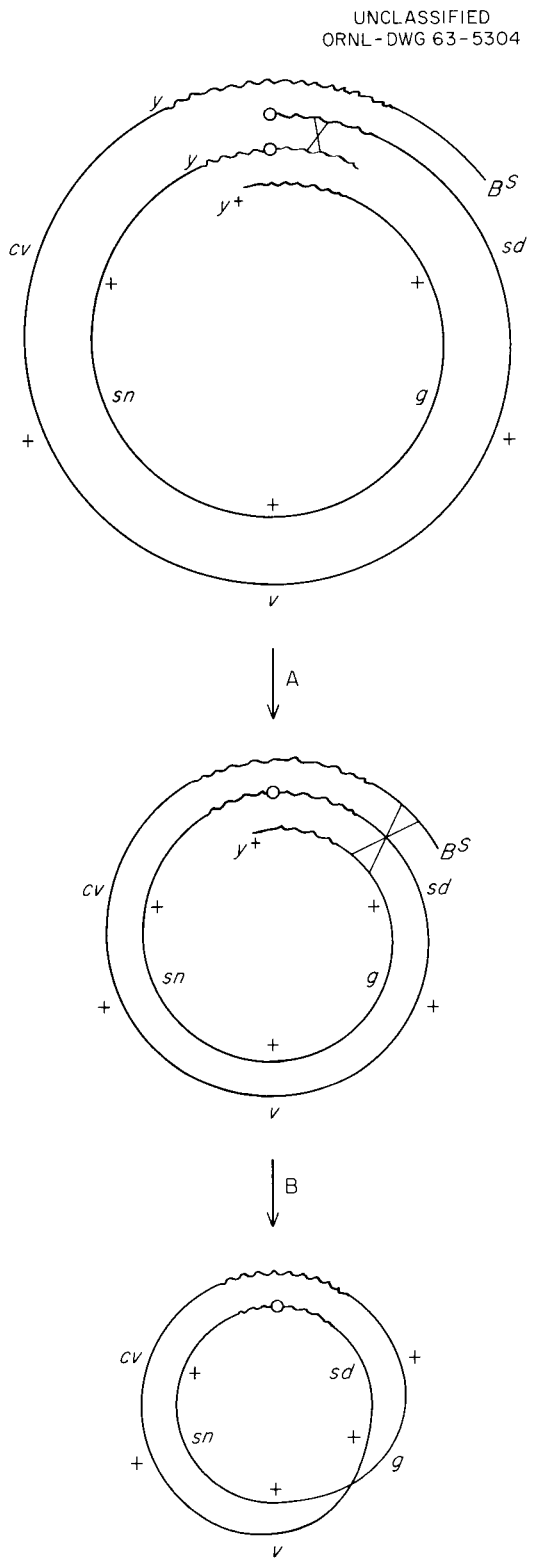

Fig. 6. Steps Utilized To Produce a Tandem Ring Compound X Chromosome.

The products of no, one, and two exchanges in a TRX chromosome are shown in Fig. 7, and the frequencies with which these products are recovered are shown in Table 11. When a dyad is composed of elements of unequal size, the smaller one is recovered with a frequency, c, which is greater than 0.5, and the larger is recovered with a frequency 1 − c (ref 2). Two types of asymmetrical dyads are produced from the TRX: One is composed of a single ring and a double ring and has a coefficient of nonrandomness of c_1; the other is composed of a single ring and a triple ring and has a coefficient of nonrandomness of c_2. The triple rings and the asymmetric bridges (i.e., 3:1 and 2:1) produce inviable zygotes. Novitski[3] has postulated

[2]E. Novitski, *Genetics* **36,** 267 (1951).

[3]E. Novitski, *J. Cellular Comp. Physiol.* (Suppl. 2) **45,** 151 (1955).

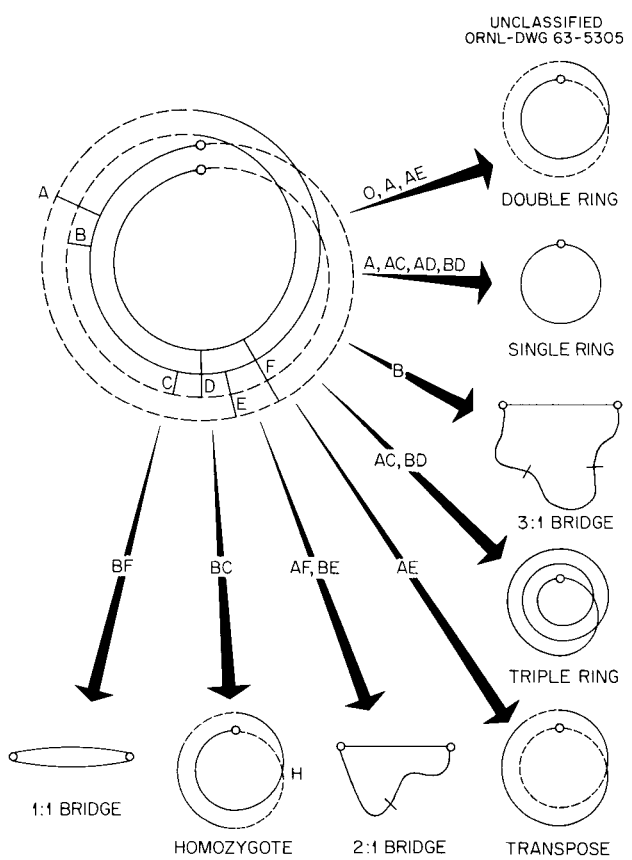

Fig. 7. Products from No- (O), Single- (A and B), and Double-Exchange Tetrads (AC, AD, AE, AF, BC, BD, BE, and BF) in the Tandem Ring.

that the symmetrical double bridges produce nullo-X ova and thus produce true patroclinous males (i.e., males that receive neither an X nor a Y from their mother). Thus, we can write the following equations for the frequencies of the products recovered in terms of the number of exchanges in the tetrad, E_i, and c_1 and c_2:

$$\text{Double rings} = E_0 + \tfrac{1}{2}(1 - c_1)E_1 + \tfrac{1}{16}E_2 ,$$

$$\text{Transposes} = \tfrac{1}{16}E_2 ,$$

$$\text{Homozygotes} = \tfrac{1}{8}E_2 ,$$

$$\text{Single rings} = \tfrac{1}{2}c_1 E_1 + \tfrac{1}{8}(1 + 2c_2)E_2 ,$$

$$\text{True patroclinous males} = \tfrac{1}{8}E_2 .$$

The TRX/FR2 females (FR2 is a Y-chromosome fragment marked with y^+) produce equal numbers of eggs derived from the TRX and FR2 products of the first meiotic division; and since half of all viable products are recovered, depending on whether fertilization is effected by an X- or a Y-bearing sperm, the best estimate of the total number of ova derived from TRX-bearing secondary oocytes is the number of FR2-bearing patroclinous males. Since single rings are recovered following fertilization by both X- and Y-bearing sperm, only ring-bearing females are used in the computations.

The data that have been collected to date are as follows:

Patroclinous males	= 428 ,
Double ring females	= 85 = 0.21 ,
Homozygous females	= 11 = 0.03 ,
Single ring females	= 175 = 0.44 ,
Single ring males	= 149 ,
True patroclinous males	= 72 = 0.18 .

At this writing no transposes have been recorded; but a test cross is required to demonstrate a transpose, and very few test crosses have been completed. There seems to be an excess of true patroclinous males, both because $8 \times 0.18 = 1.44$ is an inadmissible estimate of E_2 and because homozygous ♀♀ = 0.03 = $\tfrac{1}{8}E_2 \neq$ true patroclinous ♂♂ = 0.18 = $\tfrac{1}{8}E_2$. This would suggest a source

Table 11. Products of Exchange Within a Tandem Ring Compound X Chromosome and the Frequency of Recovery in the F_1

Exchange		Ootid
Region	Frequency	(Type and Frequency)
O	E_0	(1/2) double ring + (1/2) double ring
A	$1/2\ E_1$	$(1 - c_1)$ double ring + (c_1) single ring
B	$1/2\ E_1$	3:1 Bridge
AC	$1/8\ E_2$	$(1 - c_2)$ triple ring + (c_2) single ring
AD	$1/8\ E_2$	(1/2) single ring + 1/2 single ring
AE	$1/8\ E_2$	1/2 double ring + 1/2 transpose
AF	$1/8\ E_2$	2:1 Bridge
BC	$1/8\ E_2$	1/2 homozygote + 1/2 homozygote
BD	$1/8\ E_2$	$(1 - c_2)$ triple ring + c_2 single rings
BE	$1/8\ E_2$	2:1 Bridge
BF	$1/8\ E_2$	1:1 Bridge

of true patroclinous males other than symmetrical double bridges. The virtual absence of TRX/FR2 and X^c/FR2 progeny indicates that primary nondisjunction of TRX and FR2 is not a significant source of true patroclinous males. In the only other situation in which a 3X:1X asymmetrical dyad has been studied,[4] the value of $c_2 = 1.0$. Assuming this to be the case for the TRX and assuming that the estimate of $E_2 = 0.24$ from the homozygotes is correct leads to an inadmissible estimate of c_1 (2.03). It seems likely that some of the ring products are not fully recoverable and that the equations do not truly represent the situation; it may be, however, that when the scoring is completed, the proportions of the various classes will be quite different and more in agreement with expectation.

NONHOMOLOGOUS PAIRING OF COMPOUND CHROMOSOMES IN *DROSOPHILA MELANOGASTER* FEMALES

E. H. Grell

Compound chromosomes are formed by the joining of two homologous chromosome arms onto one centromere. The first and most familiar example of a compound chromosome is the attached-X. Other kinds of compound chromosomes, depending on centromere position and relative order of arms, have been synthesized. Arms of autosomal chromosomes have also been attached.

Nonhomologous pairing of chromosomes leads to nonrandom assortment of nonhomologous chromosomes. This phenomenon had not previously been studied with compound chromosomes. Compound-X chromosomes and a second chromosome pass to opposite poles of the first meiotic division spindle with a frequency of 0.8 to 0.9 in situations which allow nonhomologous pairing. A compound fourth chromosome was found to segregate from a second chromosome with a frequency of 0.95. A compound-4 segregates from a Y chromosome with a frequency of 0.988. When a compound-X and a compound-4 are available for nonhomologous pairing, they pass to opposite poles with a frequency of 0.97.

Crossing over within a compound-X does not affect the nonhomologous pairing. When three chromosomes are available, the segregation appears to be from a trivalent. One of the three chromosomes tends to go to one pole of the first meiotic division spindle, and two go to the other pole. In females with an attached-X, a Y, and an attached-4, the Y tends to go to one pole, and the other two chromosomes go to the opposite pole. In females with a Y, an attached-4, and a normal 4, the attached-4 goes to one pole, and the Y and the normal 4 pass to the other. They follow the rule stated by Grell (next abstract, this report) that the chromosome of intermediate size goes one way and the largest and smallest chromosomes go the other.

[4]L. Sandler and D. L. Lindsley, "The Meiotic Behavior of Tandem Acrocentric Compound X Chromosomes in *Drosophila melanogaster*," *Genetics*, in press.

REGULATION OF PREFERENTIAL PAIRING, TRIVALENT FORMATION, AND TRIVALENT DISJUNCTION BY CHROMOSOME SIZE

Rhoda F. Grell

Introduction. – As judged by segregation ratios, a single nonrecombinant chromosome, present in the *Drosophila melanogaster* female after exchange, acts as a univalent and assorts randomly with the bivalents present, two such chromosomes, whether homologues or nonhomologues, associate with varying degrees of efficiency and move to opposite poles whereas three nonrecombinants compete for associations with one another and may exhibit marked preferences.[5] Previous studies[6] have demonstrated that in a noncompetitive situation, the frequency of association between two nonhomologues is positively correlated with the similarity of their metaphase length. If chromosome size also serves as the basis for preferential pairing, it should be possible by maintaining two chromosomes constant and varying the size of the third to regulate as well as to predict the preferences that will be shown. The present work demonstrates that, for the system investigated, not only are pairing preferences controlled through chromosome size but that the decision as to complete bivalent or to bivalent plus trivalent formation, the frequency of trivalent formation when it occurs, and, finally, the manner of segregation of the chromosomes from a trivalent are all clearly a function of the size of the chromosomes involved.

[5]Rhoda F. Grell and E. H. Grell, *Proc. Natl. Acad. Sci. U.S.* **46**, 51 (1960).

[6]Rhoda F. Grell, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 47–49.

Methods. – The three nonrecombinant chromosomes utilized for the studies are a free fourth chromosome, part of a 3;4 translocation designated T_4, and a series of free X-duplications. The X-duplications, whose metaphase length varies from ~0.3 to 4+ as compared to chromosome four (arbitrarily rated as 1.0), all carry the distal tip of X and varying amounts of X proximal heterochromatin. The T_4 chromosome carries the centromere and proximal heterochromatin of 4 and the distal euchromatin of 3R and is considerably larger than any of the duplications. The T_4 is nonrecombinant in excess of 98% by virtue of the presence of a dominantly marked transposition, T_p(3R)Vno, in its nontranslocated homologue while the duplications and chromosome 4 are virtually always nonrecombinants. Nondisjunction frequencies were measured for the three chromosomes taken two at a time. Association frequencies were calculated from nondisjunction frequencies by the expression $a = 1 - 2n$, where a is percent association and n is percent nondisjunction.

Results and Discussion. – The effect of a competitor, T_4, on duplication,4 nondisjunction is shown in Fig. 8. For duplications most similar in size to chromosome 4 (0.7–1.6), the competitor has little, if any, effect on nondisjunction; whereas with duplications of lesser or greater size, T_4 markedly increases nondisjunction. The extent of increase is essentially a function of the change in size of the duplication with respect to chromosome 4. The asymmetry of the curve probably indicates that the closer the duplication approaches T_4 in size, the greater the ability of T_4 to interfere with duplication,4 association.

Concurrent examination of the frequencies of T_4,4, T_4,duplication and duplication,4 nondisjunction (Fig. 9) permits a more comprehensive interpretation of the data. For the smallest duplication (~0.3), duplication,4 nondisjunction equals T_4,4 nondisjunction. As the duplication size approaches 1, duplication,4 nondisjunction decreases and T_4,4 nondisjunction concomitantly increases. At the point of apparent equality in size between the duplication and 4, their nondisjunction is minimal and T_4 assorts randomly (~50% nondisjunction) with both chromosomes. Beyond this point, i.e., as the duplication becomes larger than the 4 and begins to approach the T_4 in size, duplication,4 nondisjunction increases while nondisjunction of the duplication and T_4

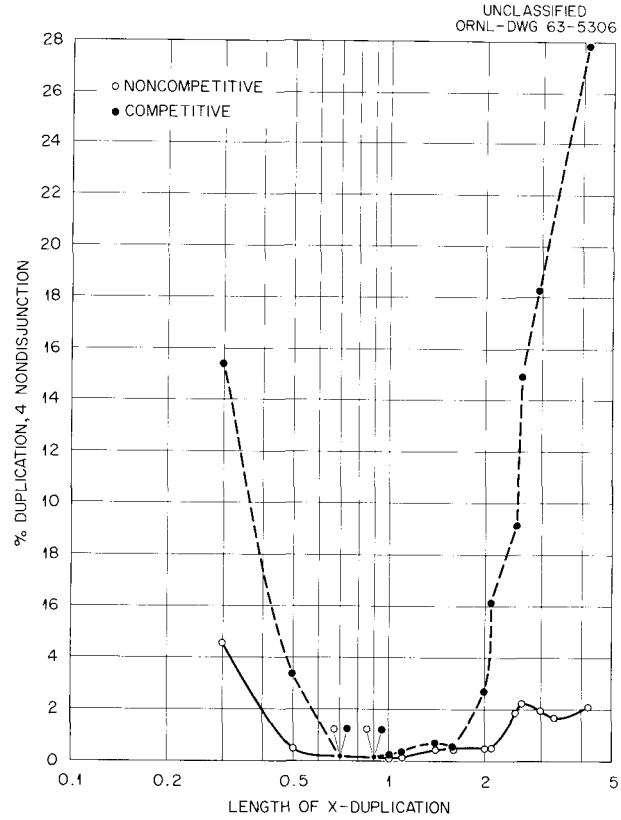

Fig. 8. Comparison of Percent Duplication,4 Nondisjunction in a Noncompetitive and a Competitive Situation.

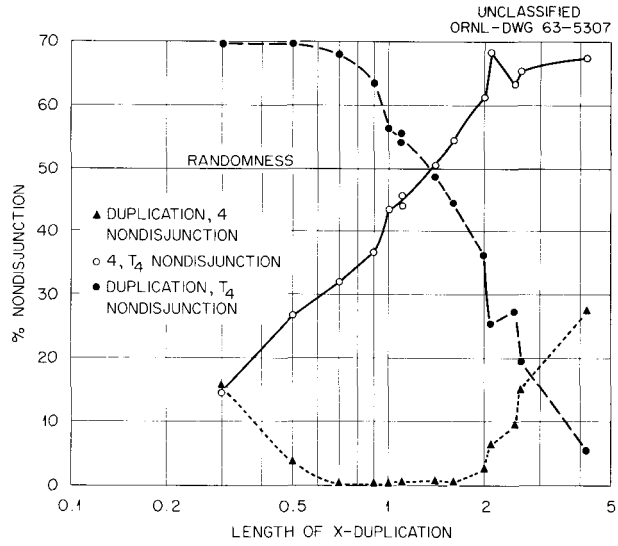

Fig. 9. Relation of Nondisjunction Frequencies to Length of Duplication.

decreases. It should be pointed out that equality of size at metaphase does not necessarily reflect equality of size at the time pairing takes place. Probably for this reason, the midpoint of the curve is not precisely at 1.0 and appears closer to 1.4.

Random assortment occurs when nondisjunction of two elements reaches 50%. Values in excess of 50% indicate recovery of two chromosomes at the same pole in excess of randomness and mean that some trivalent association has taken place. Trivalents are formed both when duplications are smaller or larger than chromosome 4. In the former case the duplication and T_4 move to the same pole in excess of 50% while in the latter, the 4 and T_4 nondisjoin more than 50%. The frequency of trivalent formation with the different duplications is indicated by association values higher than 100% for any chromosome (Table 12) and shows a positive correlation with the proportional size difference between the duplication and chromosome 4. Identity in size between the two is, therefore, expected to eliminate trivalents. The closest approximation to this point occurs with duplication No. 1337 (1.4).

A remarkable feature of the trivalent formation concerns the manner of disjunction of the elements. Association values in excess of 100% for a chromosome (Table 12) expresses the frequency that this chromosome has simultaneously associated with the other two and directed them to the same pole. With duplications smaller than 1.4, the directing element is the fourth chromosome, and for those greater than 1.4 the directing element is the duplication. Thus, it is always the element of *intermediate* size that directs the smaller and larger chromosomes of the trivalent to the same pole. The point at which the shift in directing elements occurs necessarily corresponds to the point at which trivalent association is eliminated and is probably the most sensitive indicator of equality in chromosome size at distributive pairing.

These results permit one to conclude that the preferences exhibited among chromosomes at distributive pairing, the frequency of trivalent formation, and the type of disjunction from trivalents are all a consequence of the size relations of the chromosomes involved. The principles underlying the behavior of the chromosomes in these experiments may very well apply to all the chromosomes of the *Drosophila* genome and could conceivably be extended to the behavior of chromosomes of other organisms.

Table 12. Association Frequencies[a] of Chromosome 4, T_4, and X-Duplication as a Function of X-Duplication Length

Duplication No.	Duplication Length	Chromosome 4 Association (%)	Duplication Association (%)	T_4 Association (%)	Total
1187	~0.3	139.5	69.2	70.2	2755
1162	0.5	139.8	93.4	46.4	3172
816	0.7	135.3	99.3	36.0	2481
1204	0.9	126.5	99.8	26.6	2376
1193	1.0	113.1	99.9	13.1	2744
1339	1.1	111.3	99.3	11.9	2418
1144	1.1	108.0	99.5	8.5	2667
1337	1.4	98.7	100.9	2.2	1682
1186	1.6	99.0	109.5	10.6	3714
1346	2.0	94.6	122.3	27.7	2041
1328	2.1	87.7	137.1	49.4	648
1488	2.5	81.8	127.0	45.2	1276
1343	2.6	70.2	131.5	61.5	982
856	3.0	63.4	137.0	73.5	257
3	4+	44.4	135.0	90.6	680

[a]Based on minimal trivalent formation.

MOSAICS INVOLVING ARISTAPEDIA, A HOMEOTIC MUTANT OF *DROSOPHILA MELANOGASTER*

P. A. Roberts

Introduction. – A homeotic mutant alters an appendage of a segmental series from its typical form to that of some other member of the series. One such mutant is aristapedia, which transforms the arista of the antenna into a leg. Because of the drastic nature of the transformation, it has been suggested that homeotic mutants change morphological fields or "prepatterns." For this reason, it is of interest to determine whether aristapedia affects the antennal prepattern or the competence of cells to respond to an invariant prepattern.

Materials and Methods. – Genetic mosaics were produced by x-ray-induced somatic crossing over. $T(1;3)0_5$ has a small piece of chromosome 3 bearing the wild-type allele of aristapedia (ss^a, 3-58.5) inserted close to the tip of the X chromosome which also carries the wild-type allele of yellow (y, 1-0.0). Males of the constitution $T(1;3)0_5$, D/ss^a were crossed to y/y; ss^a/ss^a females. First instar larvae were exposed to 1200-r 250-kvp x irradiation, allowed to mature, and adult females were examined for antennal mosaicism. Somatic crossing over proximal to the duplication produces homozygous ss^a tissue, which is indentifiable by yellow bristles and hairs on a background of wild-type tissue with black bristles and hairs.

Results and Discussion. – Examination of 1057 translocation-bearing females disclosed 46 mosaic antennae. The majority of mosaics consisted of antennae which were leg-like proximally and arista-like distally. Even when tissues of the two genotypes are intermingled, considerable autonomy is maintained. If the mutant and wild-type alleles of aristapedia control antennal prepatterns, an antenna which is predominantly one type of tissue should have the corresponding prepattern. A small amount of tissue of the other genotype would be expected to conform to the prepattern set up by the tissue which predominates. Since the autonomy of a small amount of tissue of one genotype is maintained when surrounded by tissue of the other genotype, the mutant and wild-type alleles of aristapedia clearly do not control antennal prepatterns. It is suggested that the aristapedia alleles determine the competence of antennal tissue to respond to a prepattern resembling the primitive, unspecialized arthropod appendage, a segmented organ of a definite length.

SURVIVAL RATES FROM OFFSPRING OF *DROSOPHILA MELANOGASTER* MALES AND FEMALES CULTURED ON NONIRRADIATED FOOD OR ON FOOD IRRADIATED WITH 500 kr

R. R. Rinehart Mary C. Stewart

Introduction. – A recent report[7] has indicated that offspring from *Drosophila* grown on irradiated food may show increased mutation rates. Since this problem has implication in food sterilization techniques, it was felt necessary to retest these results. The techniques, results, and conclusions from our experiments (testing for modifications in offspring survival that might be associated with the x irradiation of food used in culturing *Drosophila* male and female parents) are briefly presented here. Since techniques are very important in these tests, the essentials of the methods employed in these experiments measuring the survival rates are presented fairly completely.

Methods. – First instar larvae from *D. melanogaster* (24- to 30-hr) (Oregon-R strain) were collected. Simultaneously, seven *Drosophila* culture vials containing enriched *Drosophila* food were irradiated with 500 kr at an exposure rate of approximately 7 kr/min. The x rays were delivered from a General Electric Maxitron x-ray machine operating at 250 kvp and 30 ma. The larvae were placed on the food no later than 5 min after completion of x irradiation. At the same time a similar group of larvae were put on nonirradiated food. No more than 100 larvae were put in any single vial to avoid possible crowding effects. The larvae from the two sets of vials were allowed to mature at 24 to 25°C and 60% relative humidity. No differences between the two groups of flies were noted in either the time necessary for pupation or eclosion. Virgin males and females were collected

[7] M. S. Swaminathan, "Incidence of Mutations in *Drosophila melanogaster* Fed on Irradiated Medium," *Science*, in press.

from the irradiated and nonirradiated food vials and were pair mated according to the mating scheme presented below:

N-♂ × N-♀	Male and female from nonirradiated food
X-♂ × N-♀	Male from irradiated food – female from nonirradiated food
N-♂ × X-♀	Male from nonirradiated food – female from irradiated food
X-♂ × X-♀	Male and female from irradiated food

The flies were transferred to new food every 12 hr, and the eggs laid during this period were counted. A ratio of the number of pupae developing from these eggs to the total number of eggs laid gave the survival rate for each test, the measure of possible damage used. Three separate series of experiments were conducted, with two investigators separately compiling data from each test.

Results. – The results of the three separate experiments are presented in Table 13. Standard errors are given for the combined data for the three experiments to aid in determining the reliability of the tests.

No significant differences are noted between any of the tests. Therefore, in this system, flies cultured on x-irradiated food appear to have no associated decrease in the survival of their offspring.

A series of tests concerning possible damage to flies grown on x-irradiated food, using sex-linked lethals as the indication of genetic damage to the population, is now in progress. These experiments should provide more critical information as to possible interactions of the x rays and the culture medium of the *Drosophila*, and possible genetic consequences.

EFFECTS OF CLARET-NONDISJUNCTIONAL ON CHROMOSOME BEHAVIOR IN *DROSOPHILA* FEMALES

D. G. Davis

Drosophila melanogaster females homozygous for ca^{nd} were tested to determine the nature and extent of chromosome nondisjunction and loss during egg maturation and embryonic development. In addition to that already known for structurally normal X chromosomes,[8] nondisjunction and loss of each of the autosomes and structurally abnormal sex chromosomes were examined. Different chromosome pairs in the egg nucleus tended to be concomitantly involved in nondisjunction more often than expected if nondisjunction were random and tended to be concomitantly lost more often

[8]E. B. Lewis and W. Gencarella, *Genetics* **37**, 600 (1952).

Table 13. Survival Rates from Offspring of *D. melanogaster* Males and Females Cultured on Nonirradiated Food or on Food Irradiated with 500 kr

Matings[a]	Experiment 1		Experiment 2		Experiment 3		Total		Standard Error
	Pupae/Eggs	Percent Survival	Pupae/Eggs	Percent Survival	Pupae/Eggs	Percent Survival	Pupae/Eggs	Percent Survival	
N-♂ × N-♀	867/990	87.6	661/725	91.2	545/619	88.0	2073/2334	88.8	±0.652
X-♂ × N-♀	507/591	85.8	633/685	92.4	753/830	90.7	1893/2106	89.9	±0.657
N-♂ × X-♀	773/894	86.5	738/799	92.4	962/1082	88.9	2473/2775	89.1	±0.590
X-♂ × X-♀	848/997	85.1	563/632	89.1	852/947	90.0	2263/2576	87.8	±0.644

[a]N parent cultured on nonirradiated food.
X parent cultured on irradiated food.

than expected if loss were random.[9] Nondisjunction and loss were then interpreted as separate and distinct events sharing the property of nonrandomness.

Analyses of nondisjunction of fourth chromosomes (ci^{D}/spa^{Cat}), structurally heterozygous X chromosomes (Ins(1)dl-49, B^{M1}, y^{2} *sc v/y*), and a reversed acrocentric compound X with a marked Y (RA, *y f*/y^{+}Y) in ca^{nd} females showed that nondisjunction occurred at the first and never at the second meiotic division. Since these paired chromosomes rarely cross over or nondisjoin spontaneously, it is likely that the type of pairing which immediately precedes anaphase I is distributive, although preceded by exchange-type pairing.[10] The results showed that nondisjunction occurred in ca^{nd} eggs irrespectively of the final type of pairing. Analyses of gynandromorphs and excess exceptional males, both of which resulted from the loss of X chromosomes, indicated that loss may occur either during meiosis or during early cleavage mitoses but probably not contemporaneously with nondisjunction.

The facts that nondisjunction and loss share the property of nonrandomness but differ in probable times of occurrence suggest a mechanism having alternate results depending upon the chromosome mechanics of the divisions involved. The results and analyses are consistent with a model based on defective spindle fibers, like those observed by Wald,[11] in maturation and early cleavage divisions of ca^{nd} eggs, wherein nondisjunction occurs at anaphase I when a bivalent fails to separate and is drawn intact to one pole and loss occurs at anaphase II and subsequent cleavage mitoses when equationally separated daughter chromosomes fail to be drawn to a pole.

[9] D. G. Davis, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 49–50.

[10] Rhoda F. Grell, *Proc. Natl. Acad. Sci. U.S.* **48**, 165 (1962).

[11] H. Wald, *Genetics* **21**, 264 (1936).

Cell Growth and Reproduction

D. M. Prescott

Cytochemistry

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J. G. Carlson[b]
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O. L. Miller, Jr.[a]
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Romance F. Carrier
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Tuneo Yamada
S. K. Brahma[g]
Günter Cleffmann[g]
Sara Eisenberg-Wieder[g]
Shuichi Karasaki[g]
Chinami Takata[g]
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Insect Genetics

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DOMINANT LETHALITY INDUCED BY RADIATION IN HAPLOID AND DIPLOID SPERM OF THE WASP *MORMONIELLA*

R. C. von Borstel R. K. Mortimer[1]

Introduction. – In yeast, more dominant lethality is induced in diploid than in haploid vegetative cells,[2] and this finding is consistent with the observed increase in x-ray sensitivity of higher ploidy cells in this organism.[3] To study dominant lethality with respect to ploidy in a higher organism, the parasitic wasp *Mormoniella* was selected. Stocks which produce normal haploid males with haploid sperm as well as fertile diploid males with 100% diploid sperm are available. The males can be irradiated and mated with normal females for an unambiguous analysis of dominant lethality in haploid and diploid nuclei.

Results. – The frequencies of dominant lethality at different doses were estimated from hatchability, D, and from the sex ratio, D'. The sex ratio changes to a higher frequency of males at higher doses since the males arise from the unfertilized eggs. The frequencies of sperm free of dominant lethals, $1 - D'$ and $1 - D$, as a function of dose are presented in Fig. 10. A higher frequency of dominant lethality in diploid than in haploid sperm is revealed.

Discussion. – As with comparable experiments in yeast, approximately one-half the dose is required to induce the same frequency of lethals in diploid as compared to haploid sperm of *Mormoniella*.

[1]Donner Laboratory, University of California, Berkeley.

[2]M. E. Owen and R. K. Mortimer, *Nature* 177, 625 (1956).

[3]R. K. Mortimer, *Radiation Res.* 9, 312 (1958).

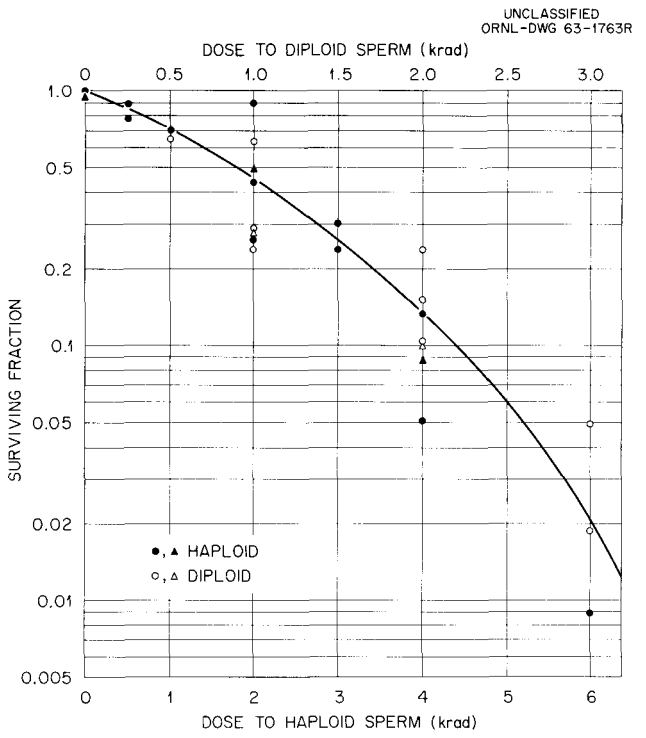

Fig. 10. Dose-Survival Curve Plotted as a Function of Ploidy X Dose for X-Irradiated Haploid and Diploid Sperm of the Wasp *Mormoniella*. The plot on the ordinate is $1 - D'$, where D' is dominant lethality as measured by sex ratio shift (circles), and $1 - D$, where D is dominant lethality deduced from hatchability of fertilized eggs (triangles).

PERSISTENT GENETIC VARIATION AFTER INBREEDING IN *HABROBRACON*

R. C. von Borstel R. E. Scossiroli[4]

Introduction. – In the Hymenoptera, fertile haploid males normally arise from unfertilized eggs, while females develop from fertilized eggs. Thus the male phenotype is a direct consequence of the genotype, and interactions among chromosome complements do not exist. Chromosome complement interaction provides a buffer against pressure from natural selection. It would seem that selection acting on haploid-diploid populations should be more effective than on diploid populations, such as those represented by *Drosophila*. An experiment was started on the wasp *Habrobracon* to investigate the presumed progress of homozygosity under inbreeding with reference to the genetic factors responsible for a quantitatively measured trait.

Results. – Since wing length was considered a suitable trait to be used for the analysis of genetic variation, a ratio of wing length to the total length of the head and thorax was taken as a measurement of the character. The *small wing, white eye* (*sw; wh*) was chosen for this experiment. Inbreeding was either father-daughter or brother-sister. The inbred lines were expanded at generations 0, 3, and 15, and selection experiments were performed.

At generation 0, both selections (for longer and shorter wings) were effective and the responses were symmetric. After three generations of inbreeding, with an expected homozygosity of 87.5%, the selection for longer wings was still effective. The selection for shorter wings showed a strong immediate response but later reached a plateau. After 15 generations of inbreeding, with an expected homozygosity of 99.99%, the selection for longer wings in the females showed a slight negative response in three replicates, while the males showed a slight positive response. Selection for shorter wings showed a response in the females like that after the third generation, while the response in the males showed a strong positive response throughout the selection period.

Discussion. – The results indicate that a certain amount of genetic variability is present in the population even after 15 generations of close inbreeding. While the heterogeneity persisted for the selection for shorter wings, homozygosity was approximated for the characters affecting the lengthening of the wings. These data indicate that the variability for longer wing length is not brought about by a high spontaneous mutation rate for quantitative characters. The genetic variation that is present in the inbred lines, as indicated by the selection for shorter wings, may be attributed to maintenance of genetic variance by persistence of heterozygosity. The sex determination mechanism of *Habrobracon* is such that heterozygosity in the region of the sex locus is mandatory. It is possible that a cluster of characters associated with a shortening of the wing is located near the sex locus.

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FURTHER ELECTRON MICROSCOPIC STUDIES ON CELLULAR CHANGES DURING LENS REGENERATION IN THE ADULT NEWT

Shuichi Karasaki

Introduction. – Changes in cell morphology during the regenerative tissue transformation of pigmented iris epithelium into lens in the adult newt *Triturus pyrrhogaster* were described briefly in a previous report.[5] In the present study, special attention was paid to the ultrastructure associated with the synthesis of macromolecules, and efforts were made to obtain quantitative as well as morphological data.

Shift in Nuclear-Cytoplasmic Ratio During the Early Phase of Lens Regeneration. – At four days or later after lens removal a conspicuous change occurs in the nuclei of the mediodorsal iris cells. The ratio of nuclear area to total cell area significantly increases in the mediodorsal iris, while no such change can be detected in the medioventral iris at any time during lens regeneration (Fig. 11). The shift in the ratio is probably due to enlargement of nuclear volume and release of pigment granules. The disappearance of the smooth-surfaced endoplasmic reticulum system may also be a contributing factor.

Nucleolar Change During the Early Phase of Lens Regeneration. – The average number of nucleoli per nucleus shows a significant increase during the first four days (Fig. 12). Nucleolar ultrastructure is similar at this stage to that of the normal iris (a fibrous component with a few internal granules). An enlargement of the nucleolus occurs during the 4- to 10-day period following lens removal (Fig. 13). This involves the acquisition of a granular component forming a cortical layer around the dense, fibrous core. The average size of the core is almost the same as that of primary nucleoli (Fig. 13). At 15 days

[5] S. Karasaki, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 59.

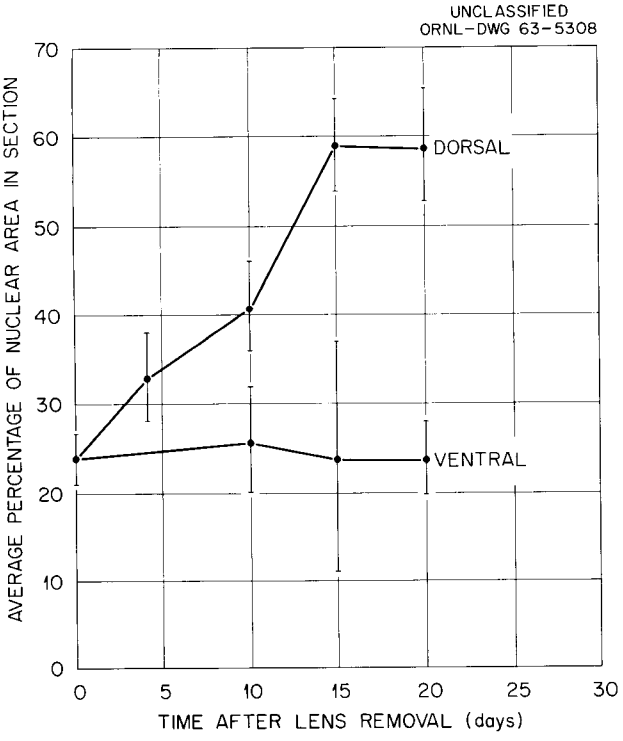

Fig. 11. Average Percentages of Nuclear Area in the Total Cell Area in the Sections of Dorsal (Upper) and Ventral (Lower) Portions of Iris During Lens Regeneration (90% Confidence Limits Are Shown).

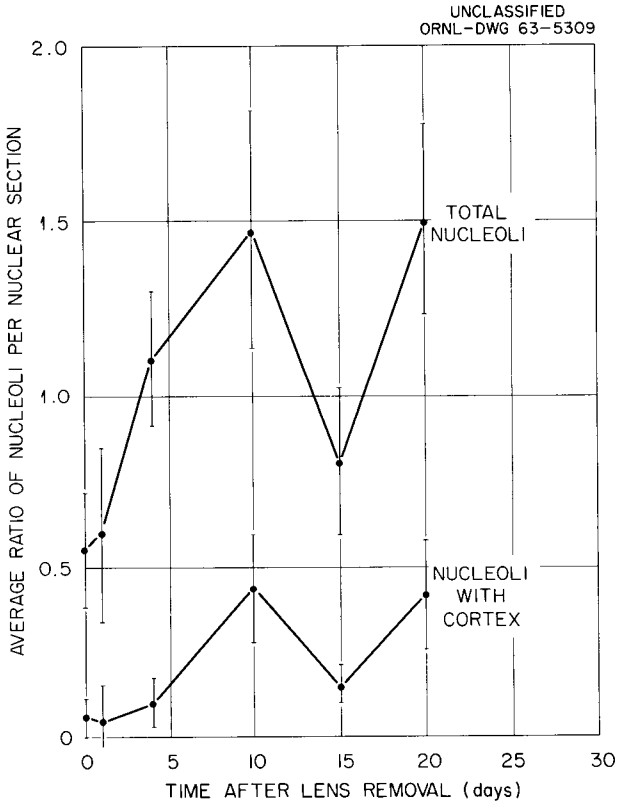

Fig. 12. Change in the Number of Nucleoli With or Without Cortex from the Sections of Dorsal Portions of Iris During Lens Regeneration (90% Confidence Limits Are Shown).

after lens removal the nucleoli again diminish in total number (Fig. 12) and in relative area of their cortical region (Fig. 13).

Frequency of Ribosomes During the Early Phase of Lens Regeneration. – Ribosomal number per unit area of cytoplasmic matrix increases during early regeneration (Fig. 14). The ribosomes are usually arranged in small clusters of 5 to 15 particles that may occasionally show rosettes or spiral patterns. Cytoplasmic basophilia, as viewed with the light microscope,[6,7] is probably due to these ribosomes. As demonstrated by autoradiographic study[8] on the same material, the distribution pattern of labeled RNA is consistent with the thesis that an acceleration occurs in the transfer of RNA synthesized in the nucleus to the cytoplasm.

[6]K. Takata, *Experientia* **8**, 217 (1952).

[7]T. Ogawa, *Embryologia* **7**, 95 (1962).

[8]T. Yamada and S. Karasaki, *Develop. Biol.* **7**, 595 (1963).

Differentiating Lens Cells. – In the regenerating lens one can observe various phases in the differentiation of lens epithelial cells to lens fiber cells, as well as the subsequent growth which lens fiber cells undergo during their gradual displacement toward the lens core. The nuclei of proliferating epithelial cells have small nucleoli with only core material, while nuclei of growing young fiber cells often exhibit large nucleoli with a regular deposition of the granular cortex. The distribution of individual cytoplasmic elements changes gradually from the equatorial lens epithelium through the zone of transition to the lens fiber cells of the interior. Toward the center of the lens, for example, the concentration of ribosomes (Fig. 14) diminishes remarkably, together with an increase of structural protein. A predominance of multiple ribosomes in the cytoplasm of epithelial cells and young fibers appears to be associated with the synthesis of intracellular protein by the lens fibers.

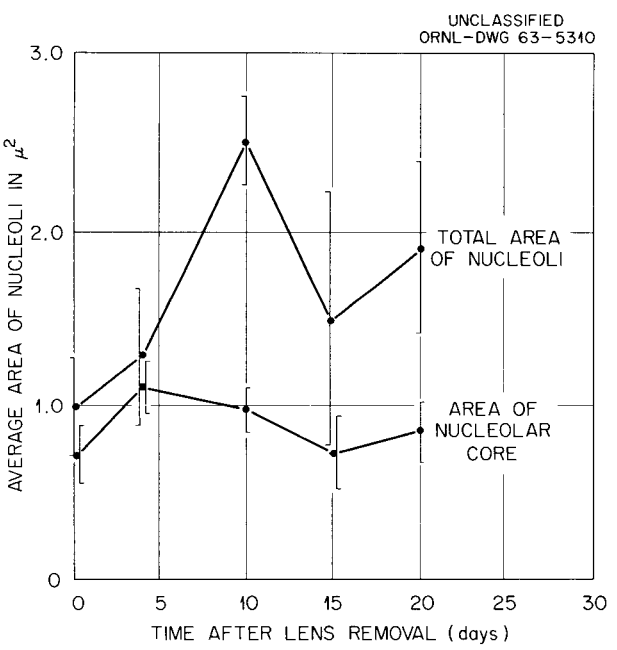

Fig. 13. Change in Sizes of Nucleoli and Their Components During Lens Regeneration (90% Confidence Limits Are Shown).

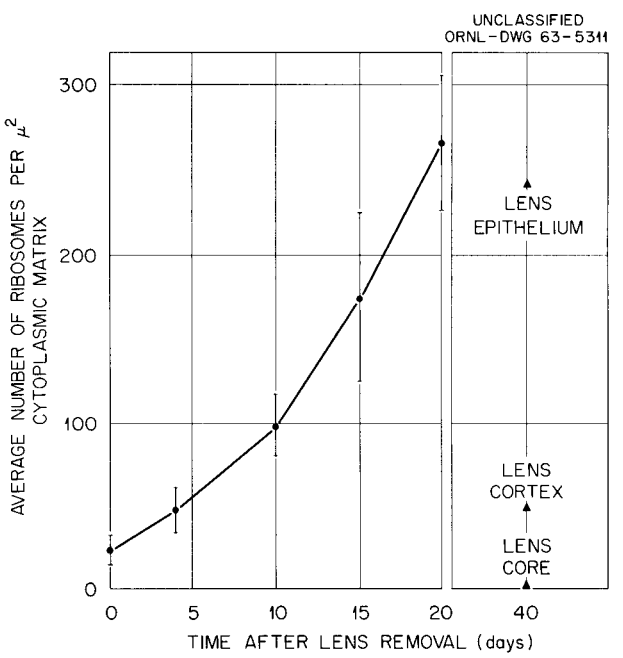

Fig. 14. Variation in the Numbers of Ribosomes in Cytoplasmic Matrix of Dorsal Iris Cells During Lens Regeneration (90% Confidence Limits Are Shown).

A PROTEIN KINASE FROM THE OVARY OF *RANA PIPIENS*

R. A. Wallace

In view of the observations that the component proteins of yolk granules are synthesized in a "soluble" form external to the oocyte, an attempt was made to explain the formation of "insoluble" yolk granules within the developing oocyte. Since it has been noticed[9] that the removal of about 25% of the phosphorus from the proteins in the yolk granule by a protein phosphatase renders the proteins soluble, the assumption was made that a partial rephosphorylation of the proteins would promote the formation of an insoluble complex. This assumption has been strengthened by the observations that protein-yolk crystals first appear and "grow" in *Rana pipiens* within the oocyte mitochondria[10] and that direct evidence for a protein-phosphorylating system was first obtained with liver mitochondria.[11] Based upon the more recent studies of Rabinowitz and Lipmann[12] on protein kinases present in yeast and brain extracts, a protein kinase has now been found in the mature ovary of *R. pipiens*.

The enzyme was most conveniently obtained from extracts of acetone powders. A four-step purification procedure involving ammonium sulfate precipitations and DEAE chromatography was developed and a 400-fold purification with a 25% yield was obtained. An important aspect of the purification procedure was the complete elimination of endogenous substrate (probably yolk proteins).

The purified enzyme promoted the steady transfer of the terminal phosphate of ATP to amphibian phosvitin over at least a 3-hr period. A strictly linear relation between phosphate incorporation and enzyme concentration was also demonstrated. In one experiment a 10% increase in the phosphorus content of phosvitin was obtained. The added phosphate (from terminally labeled ATP) was quantitatively released as labeled Pi when the protein was placed in normal NaOH at 100°C for 10 min, thus suggesting that a typical protein-phosphorus bond was present. Incubation in normal HCl or water had little or no effect.

The ovarian protein kinase reaction is stimulated optimally by phosphate at concentrations of 40 m*M*, similar to what has been observed for the brain enzyme. The pH optimum for the phosphorylation of phosvitin is between 8 and 9. Since this corresponds with the range in which an internal phosphate group migration has been observed for phosvitin,[13] the acceptor ability of substrate protein may play an important role in the phosphorylation process. The enzyme can also promote the phosphorylation of lipovitellin (phosvitin and lipovitellin are the only proteins present in the yolk granule)[14] and casein but no other proteins tested to date (plasma albumin, egg albumin, ribonuclease, chymotrypsinogen, trypsin, etc.). Inhibitors of the enzyme include *p*-mercuribenzoate and certain divalent cations (Co^{2+}, Cu^{2+}), but not iodoacetamide, dinitrophenol, Ca^{2+}, and Fe^{2+}.

The purified enzyme will now be used in further studies involving the phosphorylation of yolk proteins and the relation between the extent of phosphorylation and the peculiar dissociation-solubility properties exhibited by these proteins.

HISTOLOGICAL AND INTRACELLULAR LOCALIZATION OF PROTEIN SYNTHETIC ACTIVITIES IN THE LENS REGENERATING SYSTEM

Tuneo Yamada Chinami Takata

Introduction. – In an autoradiographic study of the transformation of iris into lens in Wolffian lens regeneration of adult *Triturus viridescens*, a strong enhancement of uptake of H^3-leucine into the transforming tissue was found to occur following an enhancement of nuclear RNA synthesis.[15] To determine whether or not this enhancement of protein synthesis is locally connected with the area of iris which is directly

[9] S. Nass, *Biol. Bull.* **122**, 232 (1962).

[10] R. T. Ward, *J. Cell Biol.* **14**, 309 (1962).

[11] G. Burnett and E. P. Kennedy, *J. Biol. Chem.* **211**, 969 (1954).

[12] M. Rabinowitz and F. Lipmann, *J. Biol. Chem.* **235**, 1043 (1960).

[13] G. Taborsky and C. C. Allende, *Biochemistry* **1**, 406 (1962).

[14] R. A. Wallace, *Biochim. Biophys. Acta* **74**, 505 (1963).

[15] T. Yamada, C. Takata, and M. E. Roesel, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 56.

involved in tissue transformation, a comparative autoradiographic study of uptake of H^3-leucine was made of one part of a lens-removed eye which regenerates lens (dorsal part of iris) and another part which does not (ventral part of iris). Fluorescent antibody studies of lens antigens in the present system show that they are mostly, if not exclusively, localized in the cytoplasm.[16] This poses the question of whether or not the radioactivity detectable with our autoradiographic technique is localized in the cytoplasm of the cells involved in tissue transformation. To answer the question, we noted the percentage of cytoplasmic radioactivity over the total tissue radioactivity for different stages of regeneration.

Histological Localization of Uptake of H^3-Leucine. – When the ratio of grain counts per unit area of tissues between the dorsal iris of lens-removed eye and the dorsal iris of the unoperated eye of the same individual was calculated for different stages of regeneration, a gradual increase in the ratio with the progress of regeneration was indicated (Fig. 15). Comparable data obtained for the ventral iris of the corresponding eyes indicate an initial increase in ratio, which is not significantly different from that of the dorsal iris, followed by a decrease to a lower level, which itself is significantly higher than the normal ventral iris level (Fig. 16). Tissue transformation, therefore, apparently is correlated with an intense enhancement of protein synthetic activities. The data suggest, on the other hand, that the fraction of protein synthesis which occurs at the earlier phase of regeneration may not be directly involved in tissue transformation, because it is observed both in the transforming and non-transforming systems. The suggestion is in line with the results obtained with immunochemical studies of lens specificity of the same system, indicating that proteins synthesized at an early phase of regeneration may not carry lens specificity.[16]

Intracellular Localization of H^3-Leucine Uptake. – The percentage of grains over the cytoplasm to those over the total dorsal tissue was calculated at various stages of regeneration using autoradiographic sections of tissues fixed

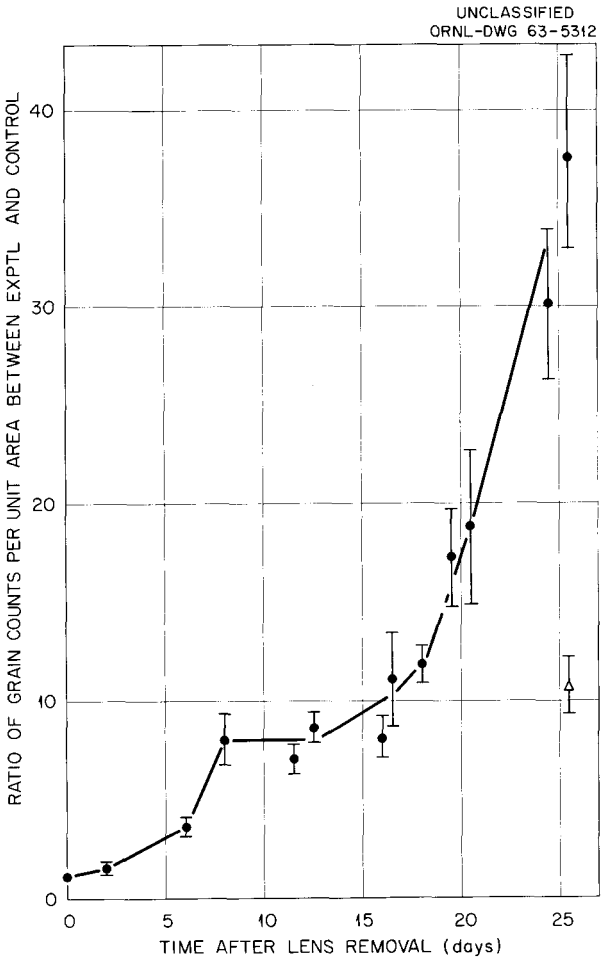

Fig. 15. Change in the Ratio Between Grain Counts per Unit Area of the Regenerating System and Those of the Control During Lens Regeneration.

3 hr after injection of H^3-leucine. As Fig. 17 indicates, an initial decrease of the value is followed by an increase. The early phase of the increasing period coincides temporally with acceleration of transfer of RNA synthesized in the nucleus into the cytoplasm[15] and with the increase in the ribosome number per cytoplasmic area.[17] A comparison of the result with immunochemical data[16] suggests that a part of protein synthetic activities demonstrated with the present technique is connected with production of a lens-specific cell component.

[16] C. Takata, J. F. Albright, and T. Yamada, this report.

[17] S. Karasaki, this report.

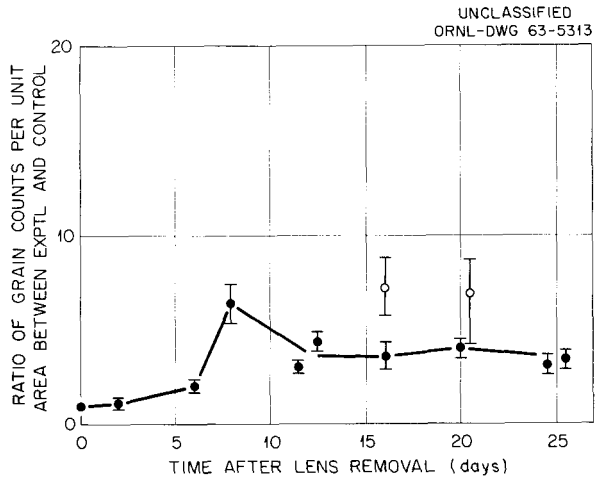

Fig. 16. Change in the Ratio Between Grain Counts per Unit Area of the Non-regenerating Part of the Iris (Ventral Iris) of the Operated Eye and Those of the Control During the Time Lens Regeneration Is Occurring in the Dorsal Part of the Iris.

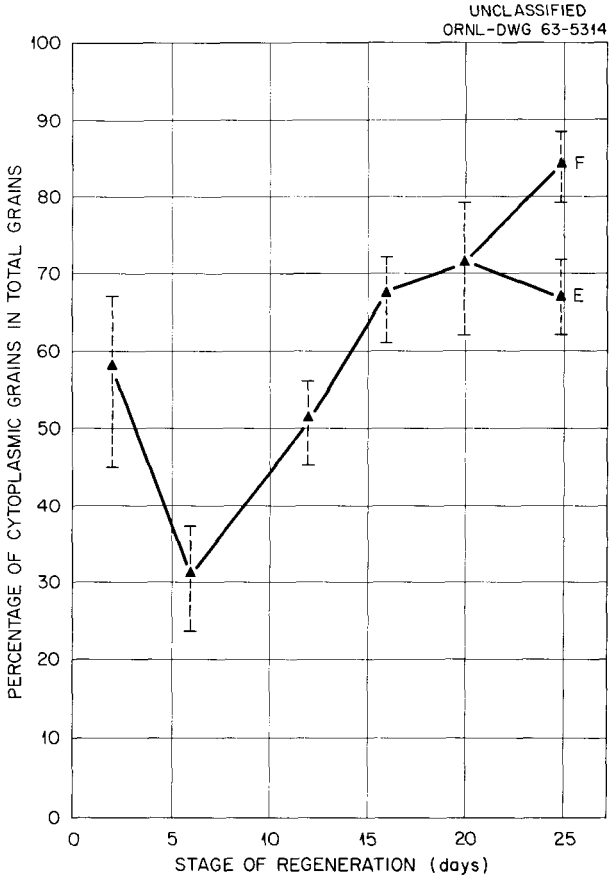

Fig. 17. Change in the Percentage of the Cytoplasmic Grains Against the Total Dorsal Tissue Grains in the Regenerating System.

IMMUNOFLUORESCENT STUDY OF LENS ANTIGENS IN DEVELOPING NEWT EMBRYOS

Chinami Takata J. F. Albright[18]
Tuneo Yamada

Introduction. – In relation to the problem of cellular differentiation, it is of some interest to learn if organ specific proteins are present in the embryos prior to morphological differentiation. We have tried to determine the time of appearance and localization of the lens antigens in the development of the lens in the newt *Triturus viridescens* by the immunofluorescent technique.

Method and Results. – Antilens serum and normal rabbit serum were prepared as described elsewhere.[19] A series of embryos, from the early tail-bud stage to the larval stage, was stained with antilens and normal rabbit serum conjugated with fluorescein isothiocyanate. In the early tail-bud embryo the lens placode did not show fluorescence after treatment with fluorescent antibody. In the later stages, when the lens vesicle is formed, fluorescence was found localized in the cytoplasm of the cells in the inner layer of the lens vesicle facing the optic cup. The cells showing positive reaction correspond to the primary lens fiber cells. The fluorescence of the lens vesicle increased its intensity, with the development of definitive lens fiber cells. In all sections the optic cup was not stained with fluorescent antibody.

Discussion. – The present experiments demonstrate that lens-specific antigens appear in the cytoplasm of primary lens fiber cells between the lens placode stage and earliest lens vesicle stage. The time of first appearance of lens antigens approximately coincides with that determined by Ten Cate and van Doorenmaaren[20] with the precipitin technique. Our results suggest that the appearance of specific lens antigens is correlated with morphogenetic events occurring in the ectoderm cells reacting to the lens-inducing influence of the optic cup, but not with inducing activities of the optic cup.

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[18]Radiation Immunology Group.

[19]C. Takata, J. F. Albright, and T. Yamada, this report.

[20]G. Ten Cate and W. J. van Doorenmaaren, *Koninkl. Ned. Akad. Wetenschap. Proc., Ser. C* **53**, 844 (1950).

LOCALIZATION AND PROGRESSIVE APPEARANCE OF LENS ANTIGENS IN TRANSFORMATION OF THE IRIS INTO THE LENS

Chinami Takata J. F. Albright[21]
Tuneo Yamada

Introduction. – When the lens in the adult newt is completely removed, the cell population at the dorsal rim of the iris transforms into the lens. Autoradiographic study[22] indicates that an enhancement of protein synthesis occurs in the lens regenerate beginning the 7th day after lens removal.

A study of appearance and localization of lens-specific antigens during lens regeneration was carried out using immunofluorescent, immunoelectrophoretic, and agar diffusion techniques.

Materials and Methods. – The antilens serum was prepared by injecting rabbits with adult *Triturus viridescens* lens homogenate in 0.15 *M* NaCl. The serum from normal healthy rabbits was used as the control. The gamma-globulin fractions from each serum were conjugated with fluorescein isothiocyanate by the method of Marshall *et al.*[23] Normal lens, normal iris, lensless eyeball, regenerating lens of various stages, blood serum, and liver of *T. viridescens* were used as sources for antigens. The method introduced by Sainte-Marie[24] was adopted for the histological procedures for immunofluorescence.

Results. – The fluorescent antibody did not stain any tissues other than the lens and the surface of corneal epithelium. Further, from immunoelectrophoresis and agar diffusion, it was found that the extracts of normal lens-less eyeball, iris, liver, and blood serum did not produce precipitin bands.

The first appearance of lens antigens in the regenerating system was observed in the cytoplasm of a few cells of the inner layer of the regenerate on the 13th to 15th day after lens removal by the immunofluorescent technique. Subsequently, fluorescence was found clearly localized in the cytoplasm of all lens fiber cells but was absent in the lens epithelium cells. In 20-day lens regenerate the antigen-antibody reaction was first observed by agar diffusion test and by immunoelectrophoresis. The number of antigen-antibody complex progressively increased with the process of the regeneration as shown in Table 14.

[21]Radiation Immunology Group.

[22]T. Yamada, C. Takata, and M. E. Roesel, this report.

[23]J. D. Marshall, W. C. Eveland, and C. W. Smith, *Proc. Soc. Exptl. Biol. Med.* **98**, 898 (1958).

[24]G. Sainte-Marie, *J. Histochem. Cytochem.* **10**, 250 (1962).

Table 14. Reaction of Lens Extracts with Lens Antibody

Methods / Antigens	Agar Diffusion: Number of Precipitin Bands	Immunoelectrophoresis: Number of Precipitin Bands
Normal lens	6 (A,B,C,D,E,F)	8 or 9 (a,b,c,d,e,f,g,h) or (a,b,c,d,e,e′,f,g,h)
12-day regenerate		0
20-day regenerate	3 (B,C,E)	3 (b,c,e)
25-day regenerate	4 (A,B,C,E)	5 (a,b,c,e,h)
30-day regenerate	5 (A,B,C,D,E)	7 (a,b,c,d,e,e′,h)
35-day regenerate	6 (A,B,C,D,E,F)	7 (a,b,c,d,e,e′,h)
40-day regenerate	6 (A,B,C,D,E,F)	7 (a,b,c,d,e,e′,h)
45-day regenerate	6 (A,B,C,D,E,F)	7 or 9 (a,b,c,d,e,g,h) or (a,a′,b,b′,c,d,e,e′,h)

Conclusions. – Appearance of lens antigens, as revealed by the fluorescent antibody study, coincides in time and site with appearance of lens fiber cells in the regenerating cell population. The pattern of lens antigens shows a progressive change with the process of lens regeneration and becomes closely comparable to that of the normal lens at about the 35th day of lens regeneration.

METABOLISM OF PIGMENT CELLS IN DIFFERENT FUNCTIONAL STATES

Günter Cleffmann

Rhythmically functioning cells are particularly suitable for the investigation of the mechanisms controlling the function of differentiated cells. In these cells the events and conditions characterizing the periods of the rhythm can be studied. A clearly defined physiological rhythm is found in the hair bulb pigment cells of agouti mice. These cells produce and "secrete" yellow melanin at a certain period of the hair development and black melanin during the rest of the time. The cell metabolism of these cells in relation to the rhythm has been studied by autoradiography.

Three groups of labeled compounds were injected into young agouti mice: amino acids that are not specifically involved in melanogenesis (C^{14}-serine, C^{14}-leucine); melanin precursors (C^{14}-tyrosine, C^{14}-DOPA); and inhibitors of the melanin-forming enzyme tyrosinase (S^{35}-cysteine, S^{35}-glutathione; as control S^{35}-sulfate). In autoradiographic sections prepared by a routine procedure, silver grains were counted per unit area. The number of grains was divided by the number of grains over the epidermis to get comparable figures (Fig. 18).

The yellow and black pigment cells take up the same amount of serine and leucine as the epidermal cells, indicating that the metabolic activity is not generally altered in either stage. Melanin precursors, however, are taken up by yellow pigment cells much less than by black pigment cells. This is in agreement with the finding that the amount of pigment formed by the yellow cells is reduced. Sulfhydryl compounds, the inhibitors of melanogenins, are incorporated into pigment cells more intensely in the yellow stage than in the black stage. This may account for the inhibition of melanin formation in yellow cells. Sulfhydryl compounds are also incorporated in yellow melanin granules to a greater extent and extruded with them from the pigment cells.

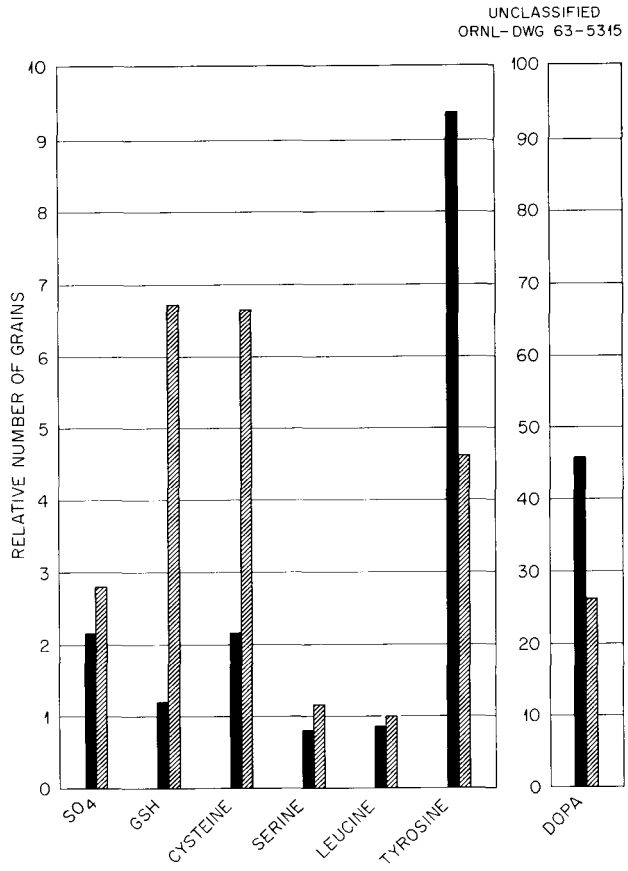

Fig. 18. Relative Grain Number for the Uptake of Different Labeled Compounds into Agouti Pigment Cells in the Yellow Phase (Hatched Column) and in the Black Phase (Solid Column). *Ordinate: ratio of grain number* over pigment cells and grain number over epidermal cells. The values for glutathione, cysteine, tyrosine, and DOPA are significantly different between black and yellow cells with 95% confidence. There is no significant difference in the values for serine and leucine. The 95% confidence limits for SO_4 are: yellow, 2.3 – *2.8* – 3.6; black, 1.7 – *2.1* – 2.7.

The reported changes in cell metabolism are specific for the action of the allele for agouti coloration, because they are not found in the pigment cells of non-agouti animals. The black non-agouti pigment cells have the metabolic properties similar to those of the agouti pigment cells in the black phase.

MACROMOLECULAR EVENTS LEADING TO CELL DIVISION IN *TETRAHYMENA PYRIFORMIS* AFTER REPLACEMENT OF REQUIRED PYRIMIDINES

I. L. Cameron D. M. Prescott

Introduction. – *Tetrahymena pyriformis* has an absolute requirement for pyrimidines in its growth medium. Earlier work by Lederberg and Mazia[25] showed that pyrimidine starvation led to rapid loss of cellular RNA while cellular protein remained constant for two to three cell generations before it, too, decreased and the cells no longer divided. Their work showed further that pyrimidine replacement led to RNA synthesis at a faster rate than protein synthesis, and they suggested that further analysis of this period should prove especially interesting. This report deals with this replacement period in terms of cellular RNA, protein, and DNA synthesis which leads to a rather synchronous burst of cell division in 6 to 8 hr.

Methods. – All cell division in mass cultures of *Tetrahymena* was arrested by 72 hr of pyrimidine starvation. Protein and RNA synthetic rates were measured by counting autoradiographic grains over individual cells after exposing samples of the cells for 15 min to H^3-leucine and H^3-uridine, respectively, at intervals after pyrimidine replacement. Other samples were removed and briefly exposed to H^3-thymidine to determine when the cells began nuclear synthesis of DNA. Cell division was followed by periodical counting of the cells grown in capillary culture tubes.

Results and Discussion. – Cellular RNA synthesis began to increase in rate immediately after pyrimidine replacement and continued to rise for at least 150 min. However, protein synthesis remained at a low level and did not begin to rise until 60 to 75 min after pyrimidine replacement. The percentage of cells with DNA-labeled macronuclei began to increase at 150 min; at 240 to 270 min 98% of the macronuclei were labeled, and the percentage then began to decrease from 300 to 390 min, at which time a low of 30% of the macronuclei were labeled. At 360 min the first cells began to divide, and at 420 min 50% had divided; however, it took until 540 min before all cells had divided.

This sequence of macromolecular synthetic events is similar to those described for a "shift up" in bacterial growth rate; however, this study relates the synthetic events to individual cells, and it is clear that essentially all the cells are synchronized in terms of their chemical response. It is evident that the responsiveness of RNA synthetic rate to the chemical environment is more rapid and may therefore reflect the messenger and ribosomal increase necessary to support a more vigorous protein synthesis and cell growth rate. There is the possibility that this system of cell synchronization could lend itself to detailed biochemical analysis of both the cellular regulation of growth as well as the impending cell division.

[25] S. Lederberg and D. Mazia, *Exptl. Cell Res.* **21**, 590 (1960).

INITIATION OF MITOSIS IN RELATION TO THE CELL CYCLE AFTER FEEDING STARVED CHICKS

I. L. Cameron Günter Cleffmann

Introduction. – Several studies have shown that when body cells are stimulated to proliferate, their first recognizable response is to undergo DNA synthesis prior to cell division. Therefore, most cell populations whose mitotic rate can be stimulated are stalled or held in the pre-DNA synthetic period (G_1). Gelfant,[26] investigating mouse epidermal cells, has challenged this concept by concluding that there is a population of cells fully prepared to divide but blocked in the post-DNA synthetic period (G_2) until they receive an appropriate stimulus to divide.

[26] S. Gelfant, *Exptl. Cell Res.* **26**, 395 (1962).

This study was designed to determine where in the cell cycle that cells are held up. Our procedure was to feed newly hatched chicks starved for 2 to 3 days because feeding after inanition stimulates mitotic activity in mammals and chickens. After this treatment, events of the cell cycle (division, DNA synthesis) were followed by routine histologic and autoradiographic preparations of the duodenal and esophageal epithelia taken from individual animals killed at intervals after feeding. Rate of cell division was determined by mitotic index, and DNA synthetic activity was determined by autoradiographically observing the percentage of cells which incorporated H^3-thymidine shortly after injection. The duration of cellular DNA synthesis (S period) was also measured to assess the effect of starvation on the S period itself, as well as to describe more exactly the temporal parameters of the cell cycle in those cell populations under study.

The rationale for this procedure is that a population of cells initiated from G_1, for example, will not cause an increase in mitotic index until at least the time needed for S and G_2 has passed. If, on the other hand, a population of cells is initiated from G_2, they will reach mitosis much earlier. When a group of cells is initiated from G_1, the increase in number of labeled cells will occur prior to the increase in mitotic activity, whereas by initiation from G_2 the mitotic activity will increase first.

Results. – In the epithelial cells of the starved animals (body temperature 37°C), S is 7 hr vs 5 to 6 hr in normally fed animals (body temperature 40.5°C). The sequence of mitotic and DNA synthetic events in the esophageal epithelium as a function of time after the start of feeding are recorded in Fig. 19 (see legend for details). The same type of data was collected for the duodenal epithelium.

There appears to be a difference in the behavior of duodenal and esophageal epithelia. The duodenal epithelium has only one group of "stalled" cells, which were stimulated to proceed through the cell cycle from G_1. In the esophagus there were two groups of such cells. One of them was stalled in G_1, but another group was stalled in G_2; this latter group was detected by the immediate rise in mitotic activity after refeeding.

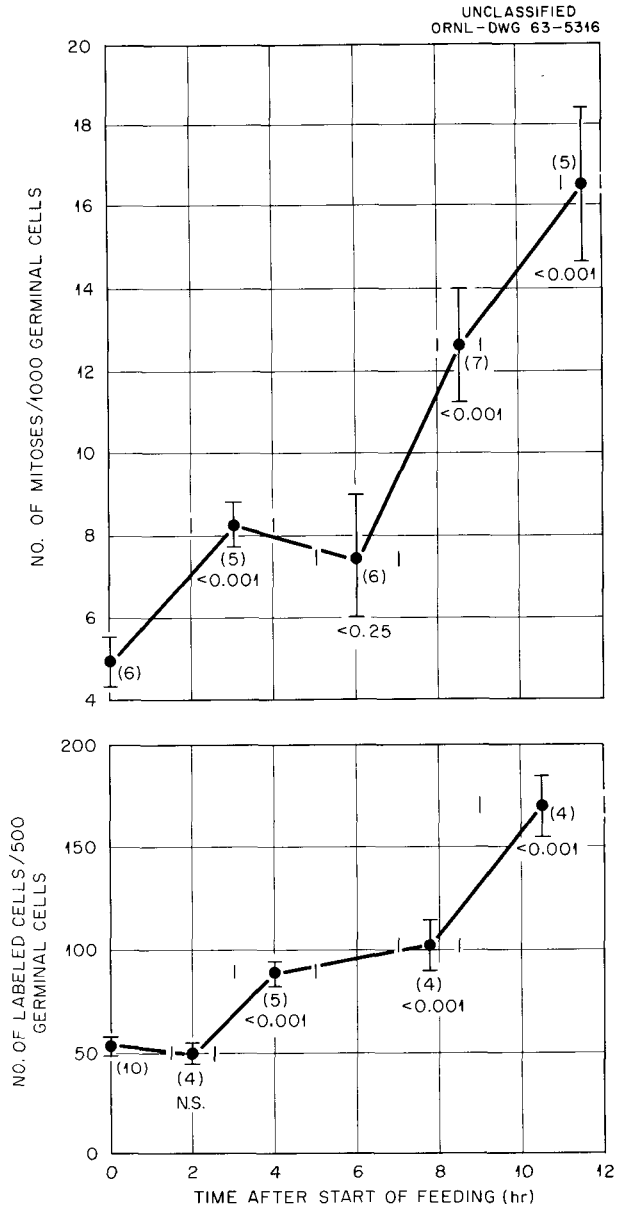

Fig. 19. Sequence of Mitotic Events (Top) and DNA Synthetic Events (Bottom) in the Esophageal Epithelium Plotted Against Time (in Hours) After the Start of Feeding of Previously Starved Animals. The average value is marked by closed circles, ●; the number in parentheses below the average is the number of animals used to determine the average. The limits of the time interval in which the animals were pooled are indicated for each point. The vertical bars indicate the standard deviation. The *P* level of significant difference between the control mean and each experimental mean is given below the average value for each group of pooled animals. N.S., not significant.

ELECTRON MICROSCOPE AUTORADIOGRAPHY OF DNA AND RNA SYNTHESES IN *EUPLOTES EURYSTOMUS*

O. L. Miller, Jr. A. Rebecca Stevens

In the macronucleus of *Euplotes*, RNA and DNA syntheses take place in mutually exclusive regions which differ with respect to the organizational state of DNA.[27] The synthesis of DNA is localized in two narrow replication bands which travel through the nucleus during the 8- to 10-hr synthesis period.[28] An electron microscope autoradiographic study was undertaken to examine more precisely the localization of RNA and DNA syntheses in the replication bands, the "chromatin bodies," and the "nucleoli."

Method. – Proliferating cells were labeled with H^3-thymidine (4 hr) or all four H^3 ribonucleosides (5 min to 21.5 hr), fixed in buffered OsO_4, and embedded in Epon 812 resin. Thin sections containing replication bands were mounted on copper-mesh grids coated with parlodion-carbon films. Subsequently, thin films of Ilford L4 emulsion were applied by the following method to specimen grids attached to microscope slides. An expandable wire loop was dipped into undiluted liquid emulsion at 30°C and then expanded 30 to 50 times its original diameter. The resulting thin emulsion film was allowed to air dry to a sticky gel and was then touched to the surface of the grids and the microscope slide. After exposures of 10 to 40 days, the autoradiographs were photographically processed by floating upside down on drops of developer and fixer. The gelatin was removed by 15-min hydrolysis on top of 0.05 *N* NaOH.

Results. – DNA synthesis occurs at the point in the replication bands where DNA undergoes transition from 150-A-diam fibers to a more finely dispersed, highly hydrated form. The region of DNA synthesis is probably less than 0.5 μ long. In the region behind the replication band, labeled DNA is primarily localized in the chromatin bodies. RNA labeling is present both in nucleoli and at the periphery of chromatin bodies.

The results support the conclusion that RNA synthesis is virtually absent in the replication bands and demonstrate that the chromatin bodies are, in fact, aggregates of DNA. Furthermore, nucleoli in the *Euplotes* macronucleus represent active centers of RNA accumulation or synthesis and in this sense are analogous to structures called nucleoli in other cells.

[27] D. M. Prescott and R. F. Kimball, *Proc. Natl. Acad. Sci. U.S.* **47**, 686 (1961).

[28] J. G. Gall, *J. Biophys. Biochem. Cytol.* **5**, 295 (1959).

DRY AUTORADIOGRAPHY OF SOLUBLE PRECURSOR POOLS IN SINGLE CELLS

O. L. Miller, Jr. G. E. Stone
D. M. Prescott

Studies of soluble precursors in single cells[29] have continued, and a dry autoradiographic technique has been developed which allows both detection and determination of cellular localization of soluble materials.

Method. – *Specimen Preparation and Extraction.* – Cells must be prepared for autoradiography by procedures that prevent loss of soluble materials and minimize intracellular displacement. Freeze-drying techniques are suitable for tissues or tissue cultures, while microorganisms can be prepared by rapid air drying of individual cells or freeze drying of wet smears. In all cases it is essential that the cells be washed free of exogenous radioactivity before drying or freezing.

The dried cells are treated in one of three ways before application of emulsion: (1) completely extracted with acid-alcohol, (2) extracted *in situ* with small drops of solvent (the pool can also be extracted with wet, gelled emulsion, as previously described),[29] or (3) not extracted. The first procedure indicates location and amount of insoluble or incorporated activity, the second detects the radioactivity within the soluble pool, and the third procedure indicates cellular location of the pool.

Preparation and Application of Dry Emulsion Films. – Thin emulsion films are prepared by dipping a large wire loop into liquid NTB3 emulsion and air drying. A slide, with the specimen placed near one end, is placed under a dried loop film so that the specimen closely underlies the

[29] O. L. Miller, Jr., G. E. Stone, and D. M. Prescott, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 62.

center portion of the film. Breath is gently blown toward the end of the slide and then toward and over the specimen until the film has adhered to the slide (tests show that the amount of moisture entering the film is not sufficient to cause detectable diffusion of labeled water-soluble compounds).

Experimental Example. – Small groups of *Tetrahymena pyriformis* were synchronized by selecting dividers from a log-phase culture. Brief incubation periods in H^3-thymidine were given at various stages of the cell cycle, followed by thorough washing with unlabeled medium and air drying on slides. When dry films were applied following complete extraction or no extraction of soluble materials, cells incubated in G_1 or G_2 showed no uptake of H^3-thymidine, while cells treated during macronuclear S showed label restricted to the macronucleus. After *in situ* extraction, cells incubated during S exhibited a halo of silver grains over the cytoplasm and around the cell in addition to macronuclear label. The results indicate that the soluble thymidine-derived pool is localized in the nucleus and the H^3-thymidine of the medium enters the pool during S but does not enter during G_1 or G_2.

DECAY OF A H^3-THYMIDINE NUCLEOTIDE POOL IN *TETRAHYMENA PYRIFORMIS*

G. E. Stone W. L. Carrier

Introduction. – Autoradiographic studies of DNA synthesis in *Tetrahymena* have suggested the presence of a soluble thymidine-derived pool existing in the cell after DNA synthesis is finished. The experiments reported here investigate whether a continuing pool of thymidine nucleotides does exist, and how it declines with time.

Methods. – A logarithmically growing culture of *T. pyriformis* was incubated for 3.5 hr (generation time 3.5 hr), centrifuged and washed three times, and resuspended in fresh medium. Samples were withdrawn from the culture, concentrated, and extracted with cold trichloroacetic acid (TCA). The cold TCA-soluble material was chromatographed on DEAE paper in 0.25 *M* NH_4HCO_3. Phosphorus-32-labeled markers (TTP, TDP, and TMP) were chromatographed at the same time for identification purposes.

Results and Discussion. – The following table shows the decrease in amount of radioactivity identified with the nucleotides.

Time after washing (min)	0	20	56	88	138	203
Nucleotide activity (% of zero time)	100	25	5	4	1	4

Ninety-five percent of the activity present at zero time (time when cells were washed free of exogenous isotope) has disappeared by 56 min. Only a trace of nucleotides is detectable during the remainder of the experiment.

The soluble pool of nucleotides disappears rapidly and cannot contribute to a continuing soluble pool from one DNA synthetic period to another. However, there seems to be a relatively larger pool that chromatographs with thymidine and decays at a much slower rate. This fraction may represent a continuing soluble pool during non-DNA synthetic periods.

CYTOCHEMICAL LOCALIZATION OF RNA SYNTHESIZED BY DNA-PRIMED RNA POLYMERASE

R. C. von Borstel D. M. Prescott
S. B. Weiss[30]

Introduction. – Primer DNA for DNA synthesis can be detected in cytological preparations by incubation of fixed cells with triphosphate deoxyribonucleotides and DNA polymerase, followed by autoradiography.[31] In an analogous system we have catalyzed polymerization of triphosphate ribonucleotides into a RNA product utilizing DNA primer present in fixed cells. For this we used the RNA polymerase of *M. lysodeikticus*,[32] which catalyzes the synthesis of a RNA product that has the overall base composition of DNA, if uracil is equated to thymine.[33]

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[30]Argonne Cancer Research Hospital, University of Chicago, Chicago, Ill.

[31]R. C. von Borstel, D. M. Prescott, and F. J. Bollum, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1961*, ORNL-3201, pp 45–46.

[32]S. B. Weiss, *Proc. Natl. Acad. Sci. U.S.* **46**, 1020 (1960).

[33]S. B. Weiss and T. Nakamoto, *Proc. Natl. Acad. Sci. U.S.* **47**, 694 (1961).

Results. – Alcohol-fixed hamster cells from tissue culture were incubated with tritium-labeled cytidine triphosphate and the RNA polymerase. The no-enzyme control showed no accumulation of triphosphate nucleotides in the fixed cells above that of the background. With the enzyme present, the cells exhibited a slight increase over background of radioactivity. In cells treated in 0.01 *N* HCl for 30 min after fixation and before incubation with the triphosphate nucleotides and the RNA polymerase, a marked accumulation of the radioactive product occurred in the cell nuclei.

Discussion. – The RNA product formed from DNA-primed RNA synthesis appears to come free from the template, allowing more product to be formed.[34] This explains the small increase over background when the experiment is carried out with undenatured DNA (alcohol-fixed cells). When the DNA is partially denatured (in our case with HCl), hybridization of the RNA product with the denatured DNA occurs.[35] This is reflected by the large amount of radioactive product retained by the acid-denatured nuclei.

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[34] E. P. Geiduschek, T. Nakamoto, and S. B. Weiss, *Proc. Natl. Acad. Sci. U.S.* **47**, 1405 (1961).

[35] R. C. Warner *et al.*, *Proc. Natl. Acad. Sci. U.S.* **49**, 533 (1963).

Mammalian Genetics and Development

Section Chief – W. L. Russell	
Genetic Effects of Radiation in Mice	**Mammalian Cytogenetics**
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EFFECTS OF RADIATION DOSE FRACTIONATION ON MUTATION FREQUENCY IN MOUSE SPERMATOGONIA

W. L. Russell Elizabeth M. Kelly

An attempt is being made to uncover the mechanism responsible for the large increase in mutation frequency obtained from 1000 r of 90 r/min x irradiation delivered in two fractions of 500 r separated by 24 hr, as compared with the result from the same total dose given in one exposure.[1] Two experiments concerned with this problem have now yielded enough data for preliminary conclusions.

In the first of these a total dose of 600 r of 90 r/min x irradiation was given in two fractions: 100 r followed by 500 r, 24 hr later. Mutation induction in spermatogonia was scored by our standard specific locus method. The purpose of the experiment was to see whether the second fraction (500 r), after a first fraction of 100 r, would raise the mutation frequency as much as if it had followed a first fraction of 500 r. To date, 31 presumed mutations have been observed in 19,839 offspring. This mutation frequency, 22.3×10^{-5} per locus, is not as high as that calculated on the basis of the above assumption, 34.7×10^{-5}. It is, however, significantly higher ($P = 0.008$) than the rate of 13.3×10^{-5} obtained in an earlier extensive

[1]W. L. Russell, *Proc. Natl. Acad. Sci. U.S.* **48**, 1724 (1962).

experiment[2] using a single 600-r exposure. Therefore, it can be concluded that this particular fractionation pattern does increase mutation frequency over that obtained from a single dose but that the increase is not as great as that forthcoming when the first part of the fractionated dose is larger.

The second experiment reversed the order of the fractions: 600 r total dose given as 500 r followed by 100 r, 24 hr later. To date 12 presumed mutations have been observed in 11,346 offspring. This frequency, 15.1×10^{-5} per locus, is not significantly different from that obtained from the single 600-r exposure (13.3×10^{-5}) nor is it significantly below the value of 18.0×10^{-5} that would have been expected if the second fraction (100 r) had produced one-fifth the augmenting effect apparently achieved by 500 r following a first fraction of 500 r.

The two experiments taken together suggest that if, as still seems likely, cell stage synchronization by the first fractionation is involved, then the degree of synchronization produced by 100 r is not as effective as that produced by 500 r.

CROSSING OVER WITHIN THE A-"LOCUS" OF THE MOUSE

Liane B. Russell Mary N. Cupp McDaniel
Florence N. Woodiel

A new mutation, a^x, derived from irradiated spermatogonia, is recessive to A^y, A^w, A, and a^t and dominant to a, the a^x/a type being distinguishable from a/a by a slightly paler belly. Homozygous a^x/a^x animals die *in utero*.

A balanced lethal $A^y/a^x \times A^y/a^x$ stock produced 1904 yellow offspring and 4 which were agouti (dark belly). Furthermore, among 260 tested yellows, one was A^y/A rather than A^y/a^x. The closely linked markers *kr* and *un* were then introduced [*kr*-(3)-*a*-(5)-*un*]. Matings of *kr* a^x +/+ A^y *un* with either a/a or A^y/a produced, among 543 young, 2 dark-bellied agouti which had received neither *kr* nor *un* from the a^x/A^y parent.

The results indicate that the agouti exceptions are recombinants between a^x and A^y and that the order is *kr*-a^x-A^y-*un*. Allowing for nondetectable recombinants, the frequency of crossing over of a^x-A^y is 0.5%. This is higher than known recombination frequencies between certain loci controlling unrelated phenotypes (*d*-*se* = 0.16%; *bp*-*a* = 0.3%).

An agouti exception was also observed in an $A^y/a \times A^y/a$ mating. Although, in this case, no linked markers were present to prove this, it appears possible that A^y is pseudoallelic to *a* just as it is to a^x.

[2]W. L. Russell, L. B. Russell, and E. M. Kelly, *Science* **128**, 1546 (1958).

A MOSAIC MOUSE PROBABLY FORMED FROM TWO MEIOTIC PRODUCTS OF OOGENESIS

Liane B. Russell Florence N. Woodiel

Introduction. – Mosaicism involving major portions of the body could come about in three ways: (1) genetic events occurring during early cleavage divisions, (2) mutation in one of the gametes, involving only one strand of a double-stranded chromosome, or (3) an abnormality in egg maturation which has been named "immediate cleavage."[3,4] The third of these, to be detectable and distinguishable from the other two, requires either the formation of a gynandromorph (which, in the mouse, is probably not easily apparent on external examination) or the presence of markers in the cross such that the mosaic can be shown to contain two meiotic products from at least one of the parents. A mosaic of this type has recently been observed in a cross involving three alleles at one locus, as well as closely linked markers.

Material and Results. – In a mating of + A^y *un we*/*kr* a^x ++ ♀ × *kr a*/*kr a* ♂, there occurred a female with yellow and black areas arranged roughly in wide transverse bands.[5] This animal is fully fertile and transmits three different alleles at the *a*-locus, namely, *a*, A^y, and a^x (21, 17, and 5 offspring respectively). Among 11 offspring tested so far, all A^y carry *un we*, and all a^x and *a* carry *kr*, but not *un we*. Analyses of mitotic metaphases of the striped female show 40 apparently normal chromosomes in 35 cells, 41 in two cells, and 39 in one cell.

[3]A. W. H. Braden, *J. Genetics* **55**, 476 (1957).

[4]Liane B. Russell, *Progr. Med. Genetics* **2**, 230 (1962).

[5]*kr* = kreisler, a behavior mutation; A^y = yellow, homozygous lethal; *a* = non-agouti (i.e., black); a^x = pale-bellied non-agouti, homozygous lethal; *un* = undulated tail; *we* = wellhaarig, a mutation affecting hair structure. Recombination, *kr*-*a* = 3%, *a*-*un* = 5%, *un*-*we* = 6%.

Discussion. – The mosaic female transmits two homologous chromosomes (LG V) present in her mother (+ A^y *un we* and *kr* a^x ++). Two types of chromosomal constitution could account for this: (1) the female is trisomic for LG V in part of her body and gonad, or (2) the female is a 2n/2n mosaic brought about by one of various possible aberrations in egg maturation and fertilization.

If (1) is the case, non-disjunction of LG V must have occurred during one of the meiotic divisions of the mother (either reductional; or equational, with crossover in reductional) to form a zygote trisomic for LG V. At an early cleavage, one of the extra chromosomes (bearing A^y) must have been lost, yielding a mosaic of trisomic and normal cells (producing yellow and black hair respectively). The only result in favor of this hypothesis is the finding of a small proportion of cells with 41 chromosomes. This proportion is, however, no higher than that normally found in tissue culture. Attempts will be made to culture skin separately from regions producing yellow and black hair.

If (2) is correct, the two different maternally contributed homologous chromosomes derive from two meiotic products of the mother. Since the father was homozygous *kr a/kr a*, and since the mosaic is apparently not a gynandromorph, no decision is possible as to whether the two maternal meiotic products were separately fertilized.

Possible pathways of origin of 2n/2n mosaics meeting other conditions are shown in Fig. 20. Pathways 1 and 2 involve "immediate cleavage" at the first meiotic division, an event found by Braden[3] to be much rarer than "immediate cleavage" at the second division (pathway 3 shown in Fig. 20). On the other hand, in all cases of the latter studied by Braden, only one of the two equal-sized cells contained a sperm; while, for the mosaic here under study, it is necessary to assume that the cell which would normally have formed the second polar body was also fertilized. Such an explanation would also have to be invoked for the human gynandromorph described by Gartler, Waxman, and Giblett.[6]

Assuming the first meiotic division to be reductional, it is necessary, in the cases of pathways 2 and 3, to assume crossing over between the centromere and the most proximal one of the markers (*kr* or *we*, as the case may be).

[6]S. M. Gartler, S. H. Waxman, and E. Giblett, *Proc. Natl. Acad. Sci. U.S.* **48**, 332 (1962).

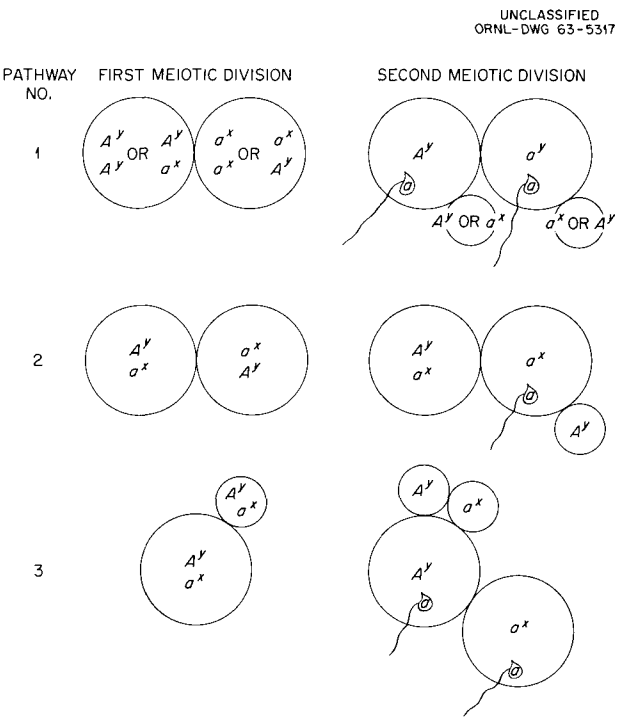

Fig. 20. Three Possible Pathways That Would Account for a 2n/2n Mosaic with Yellow and Black Fur, Resulting from a Cross of + A^y *un we*/*kr* a^x ++ × *kr a*/*kr a* and Transmitting Three Types of Chromosomes: + A^y *un we*, *kr* a^x ++, and *kr a* ++. Either the first meiotic division (pathways 1 and 2) or the second (pathway 3) is abnormal in that *both* of the resulting cells will participate in the embryo (in lieu of polar-body abstriction).

The chromosomes are designated only by their content at the *a*-locus. Retaining our earlier symbolism, the first polar body is shown at the top of the vitellus, the second at the bottom.

It cannot be definitely stated at this time which of the three possible pathways is the most likely one. Transmission ratios of the three *a*-alleles seem to point against pathway 2, which should yield, in the progeny of the mosaic, a higher proportion of a^x than of the other two types. As a matter of fact, there is a deficit of a^x progeny. If pathways 1 or 3 are correct, one would have to conclude that the meiotic product containing a^x contributed much less to gonadic tissue than did the product containing A^y. A decision between pathways 1 and 3 will never be possible, but positive proof of pathway 2 would be obtained if crossover products between + A^y *un we* and *kr* a^x ++ were to be transmitted by the mosaic female.

POPULATION DYNAMICS OF IRRADIATED TYPE A SPERMATOGONIA OF THE MOUSE

E. F. Oakberg Evelyn Clark
Patricia A. Alday

Russell observed[7] that 1000 r given in two 500-r fractions 24 hr apart results in a highly significant increase in the spermatogonial mutation rate over that observed with a single exposure of 1000 r. The rate obtained also was above that expected on the basis of extrapolation of results from 300 and 600 r. Synchronization of the spermatogonial population by the first 500-r exposure was suggested as a possible explanation of the increased mutation rate. Interaction between synchronization by the first, together with selective action of the second 500-r fraction, may also be an important factor.

The present experiments were designed to provide information on synchronization and the dynamics of spermatogonial repopulation after single x-ray exposures of 500 and 1000 r, and 1000 r divided into two 500-r fractions given 24 hr apart.

Male mice (101 × C3H), approximately 12 weeks old, were given either 500 or 1000 r in a single exposure or 1000 r in two 500-r fractions 24 hr apart. H^3-thymidine was injected 30 min before irradiation to label cells irradiated during DNA synthesis and 6 hr before irradiation to label cells irradiated during the G_2 phase. Mice were killed at 24 hr and at 5 days after irradiation.

[7]W. L. Russell, *Proc. Natl. Acad. Sci. U.S.* **48**, 1724 (1962).

Table 15. Distribution of Type A Spermatogonia Among Stages of the Cell Cycle 24 hr After 500 r

	Interphase	Prophase	Metaphase and Anaphase	Telophase
Control	49.0	36.2	1.4	13.4
500 r	90.3	9.2	0.2	0.4

Preliminary data (Table 15) indicate that 24 hr after an exposure of 500 r, the fraction of spermatogonia in interphase is much higher than in controls, with fewer cells in prophase, metaphase-anaphase, and telophase.

The decrease in percentage of labeled cells from 20.8 in the control to 8.4 for cells in S and to 8.1 for cells in G_2 observed 24 hr after irradiation suggests that the interphases present are primarily in G_1 (Table 16). However, degeneration of damaged cells still is not complete. At 5 days, when the lowest cell counts prior to repopulation are attained, the percentage of labeled cells is 1% after irradiation in S and 8.0% after irradiation in G_2. Thus, in the 1- to 5-day interval, loss of unlabeled cells and those irradiated in G_2 has been equal, but the loss from cells irradiated in S has been greater than the average for the total population. In order of increasing resistance to cell killing, the ranking is S, G_2, and G_1.

In summary, disturbances in the cell population have been observed, and the relative frequency of different cell stages 24 hr after 500 r differs from that of the control. At present, these differences are not large enough to explain the effect of dose fractionation on mutation rate. Data are now being collected on the mice given 1000 r, both as single and fractionated exposures. H^3-thymidine also has been given at different intervals after irradiation to further characterize the surviving spermatogonia and to elucidate the dynamics of spermatogonial repopulation.

Table 16. Percentage Survival of Type A Spermatogonia Irradiated in S and G_2

Dose (r)	Time Killed (days)	H^3-Thymidine I.P. 1 hr Before Irradiation (S) Labeled (%)	Survival (%)	6 hr Before Irradiation (G_2) Labeled (%)	Survival (%)
0	1	20.8		20.8	
500	1	8.4	28.7	8.1	27.9
500	5	1.0	3.3	8.0	3.3

CHEMICAL MUTATION STUDIES IN MICE

B. M. Cattanach

The procedure and initial results of experiments to test for chemically induced gene mutations in mouse spermatogonia were described in the last report.[8] The chemical under test is triethylenemelamine (TEM), and the scoring system is the specific loci method.[9]

Further results have now been accumulated and are presented in Table 17. The one mutant recovered from the group containing 0.5 mg of TEM per kg of body weight was conceived 23 days after treatment of the father. The mutation must, therefore, have been induced in a spermatozoon that was a spermatocyte rather than a spermatogonium at the time of treatment. Out of the three mutants recovered from the 4 mg/kg fractionated dose group, one died before it could be genetically tested and therefore can only be included in the calculations of the mutation frequency with some reservation.

If all mutations are scored, the mutation frequency for the three dose groups added together is 5.91×10^{-5}. If the untested mutant is excluded, then it is 4.72×10^{-5}. Both frequencies are markedly higher than the spontaneous rate[10] of 0.75×10^{-5}, and the probability that the mutants are of spontaneous origin is extremely low ($P = 0.001$ and 0.006 respectively). Therefore, TEM is undoubtedly capable of inducing gene mutations in mouse germ cells.

The data are too meager to allow any meaningful mutation frequency estimation for each dose level. However, within the range of doses employed there is no suggestion of a departure from a linear relation between mutation frequency and dose, assuming that linearity permits the calculation of a mutation frequency per dose from the pooled data. Thus, when the untested mutant is excluded, the spermatogonial mutation frequency is 2.02×10^{-5} per mg/kg per locus per gamete (upper and lower 90% confidence limits, 5.07 and 0.74). When the untested mutant is included, the frequency is 2.70×10^{-5} (upper and lower 90% confidence limits 5.80 and 1.18).

A comparison of the effectiveness of TEM and x rays tentatively may be made using the data of the highest TEM dose. The mutation frequency in the 4 mg/kg group (7.44 to 11.16×10^{-5}) lies somewhere between that produced by 300 and 600 r of x rays (8.72 and 13.29×10^{-5} respectively).[10]

In the last report[8] it was stated that the median time of recovery from the sterile period induced by 4 mg of TEM per kg of body weight was 89 days. In earlier experiments,[11] using the same dose, the recovery was observed to occur about the 62nd day. Since this result was obtained in another laboratory with a different stock of mice and with a different sample of TEM, there are many variables that might account for the discrepancy. However, the 4 mg/kg series has now been repeated, and the median time of recovery was only 67 days. The only difference between the two Oak Ridge experiments was the age of the males at the time of

[8] B. M. Cattanach, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 67.

[9] W. L. Russell, *Cold Spring Harbor Symposia Quant. Biol.* **16**, 327 (1951).

[10] W. L. Russell, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, pp 70–72.

[11] B. M. Cattanach and R. G. Edwards, *Proc. Roy. Soc. Edinburgh* **B67**, 54 (1958).

Table 17. Mutations at Specific Loci Induced in Mouse Spermatocytes and Spermatogonia by Triethylenemelamine (TEM)

Dose (mg TEM per kg of Body Weight)	Stage Treated	Number of Offspring Scored	Number of Mutants
2 + 2 (two 24-hr fractions)	Spermatogonia	3840	2 (+1?)
1	Spermatocytes	264	0
1	Spermatogonia	4339	1
0.5	Spermatocytes	667	1
0.5	Spermatogonia	2963	0

treatment. The first group, when treated, was approximately 1 yr old; the recent group, 3 to 4 months. It may be that the spermatogonia of older males are either more sensitive to killing or are replaced more slowly than those of younger animals. Evidence favoring such an age effect is found in the 0.5 mg/kg group. Here both old and young males were included in the one series. No clear sterile period was produced by this dose, but a period of low fertility was detected between days 42 and 50. In the older group of males, only 13% of the matings were fertile during this period, compared with 78% in the younger group. At other times the fertility of the old and young males was identical.

An age effect on spermatogonial killing (or replacement) does not imply that the same effect may operate on gene mutation, for these two processes seem to be independent of each other. Thus the sterile period following 1000 r is longer than that following 600 r, but the mutation frequency is lower.[10] Further, no age effect on x-ray-induced gene mutation has been demonstrated (Russell, personal communication). There is, therefore, no reason to expect that the TEM mutation frequencies may differ between experiments using old and young males.

Because of these considerations, it would seem that the 67-day sterile period is that which should be used for comparing the 4 mg/kg fractionated TEM dose with irradiation data. The 4 mg/kg sample of TEM would then be equivalent to an x-ray exposure of <600 r. In spite of the apparent independence of spermatogonial killing and mutation induction, this approximation is more in line with the comparison based on the present estimate of the mutation frequency.

Radiation Microbiology

Alexander Hollaender	
Mutagen Mechanisms – Bacterial Metabolism	**Bacterial Genetics**
G. E. Stapleton Roberto Masironi[a] J. T. Donohoo[b] Annie S. Angel H. L. Scott[c]	H. I. Adler R. Curtiss III K. A. Fry[b] Alice A. Hardigree
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BIOCHEMICAL CHANGES DURING DEVELOPMENT OF RADIORESISTANCE

G. E. Stapleton J. T. Donohoo

The nature of biochemical changes that occur in many strains of *Escherichia coli* during development of resistance in glucose-containing media is being investigated actively. Previous reports[1,2] have indicated that it is possible to divide the phenomena into phases on the bases of nutritional requirement, morphology, and macromolecular composition. We have been able to demonstrate that large cells with about twice the cellular concentration of DNA, RNA, and protein can be grown from early stationary phase cultures (which are not radioresistant) after the major pH change has occurred. Populations of these cells can, in the absence of an exogenously supplied organic nitrogen source, but in the presence of an energy source, such as glucose, develop maximal resistance within 4 to 6 hr of further incubation at pH 5.0. The development of radioresistance is pH-dependent and is essentially nonexistent at pH 7 or above.

Gross chemical analysis of fully resistant and fully sensitive cells indicates that the most striking differences in the two types of cells are the free sulfhydryl (SH) compounds found in the cold-trichloroacetic acid extract and the glycogen-like materials extractable from the cells. These compounds are significantly lower in resistant than in sensitive cells.

The sulfhydryl compounds have been of most interest, since a similar difference has been reported for radioresistant and sensitive spores of *Bacillus*.[3,4] The development of radioresistance, during sporogenesis of the spore formers, parallels in time a large reduction of free SH with a concomitant increase in disulfide in the protein of the spore. It is not improbable that mixed disulfide formation occurs with important proteins. Several investigators[4] have proposed this process as important in radiation protection afforded by

[1]G. E. Stapleton, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1962*, ORNL-3267, p 62.

[2]G. E. Stapleton, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 68–69.

[3]V. Vinter, *Folia Microbiol.* 5, 217 (1960).

[4]L. Eldjar and A. Pihl, *Radiation Res.* 9, 110 (1958).

SH protective agents. Preliminary results indicate that the free-SH content of *E. coli* does decrease with development of radioresistance, as in sporogenesis, but to date we have not been able to detect increased cystine in the protein residue after hydrolysis. More work will be required to determine whether the suspected similarity to the findings in sporogenesis are of importance in development of radioresistance in *E. coli*.

CHEMICAL PROTECTION AGAINST RADIATION DAMAGE

G. E. Stapleton H. L. Scott

A previous report[2] suggested that oxygen removal was probably an important part of the protective action of β-mercaptoethylamine (MEA) when suspensions of cells are reacted with the compound in closed vessels. It is clear that greater protection is routinely obtained with MEA at concentrations above 30 micromoles/ml than if oxygen is removed from the suspension by bubbling with oxygen-free nitrogen. Although it is known that the compound can remove oxygen from the cell suspensions, the question still exists as to the role of oxygen removal in the protective action.

Electrometric determination of dissolved oxygen, by use of the Beckman oxygen electrode inserted in a closed tube similar to that used in biological experiments, suggests that maximally effective concentrations of MEA cannot remove oxygen at a rate sufficient to explain the protection afforded. Moreover, it is possible to demonstrate almost maximal protection within 30 sec of reaction of bacterial suspensions with somewhat higher concentrations (120 micromoles/ml) even while oxygen is being vigorously bubbled through the suspension. Longer reaction times, of course, with oxygenation, lead to destruction of the compound by autooxidation.

A determined effort was made by use of a variety of techniques to demonstrate binding of MEA with the bacterial cells. Attempts to wash cells in a nitrogen-filled dry box without loss of protection were variable to unsuccessful. In most experiments, however, control samples proved the presence of oxygen in the system. These experiments suggest that if binding occurs, oxidation prevents the protection; this is partly at odds, it seems, with the aforementioned protection in the presence of oxygen bubbling for short periods. To date this apparent dilemma has not been resolved.

It has been possible to demonstrate in gas-tight tubes, of the type routinely used in bacterial irradiation experiments, that the suspension reacted with MEA becomes anaerobic long before the oxygen in the air space above the suspension is reduced. In the light of this finding, one would suspect that localized intracellular anoxia produced by MEA might play a role in protection. Two simple alternatives might lead to such an effect: (1) the localized concentrations may be higher than predictable by simple diffusion and (2) the rate of oxidation (oxygen uptake) locally may be accelerated by intracellular catalysts, such as iron or copper. Cell extracts (up to the equivalent of 10^{11} cells/ml) show no catalytic effect on autooxidation when added to MEA. Localized high concentration, which may require active binding, has not been eliminated.

REQUIREMENT FOR SULFHYDRYL GROUP IN CHEMICAL PROTECTION

Roberto Masironi G. E. Stapleton

This project was begun to test the reports[2] that disulfides of many sulfhydryl compounds are effective protective agents. Previous reports from this laboratory[1,2] claim no protection of bacterial suspension (*Escherichia coli*) by cystine or cystamine, the disulfides of cysteine and β-mercaptoethylamine (MEA). To date most of the effort has been devoted to designing experimental setups and techniques for comparing the protective action of a large number of pairs of sulfhydryl compounds and their disulfides.

As a part of this preliminary work an unexpected finding was made: in some strains of *E. coli* the size of the oxygen effect is more dependent on physiological processes than could be expected from findings reported in more than a decade of active research on this problem.

SPACE-RELATED PROJECTS

G. E. Stapleton

22-Mev Protons. – Inactivation of *Escherichia coli* by 22-Mev protons from the ORNL 86-inch cyclotron has been investigated. To date exposures have been made only in the presence of air, and the results show an RBE somewhat less than 1.0, as was previously found with exposures to 130-Mev protons from the Harvard University cyclotron. Exposures in the absence of oxygen are just beginning. Anaerobic exposures to the 130-Mev-proton beam yielded an RBE of 1.0 for inactivation as well as for mutation induction. Moreover, good chemical protection was afforded by MEA against inactivation by 130-Mev protons. Similar results are expected to be obtained with 22-Mev protons on the basis of the relative LET's of these radiations.

Similar investigations, using the high-LET ion beams from the HILAC at the University of California, are anticipated soon.

ANALYSIS OF A GENETIC LOCUS CONTROLLING CELL DIVISION AND SENSITIVITY TO RADIATION IN *ESCHERICHIA COLI* K-12

H. I. Adler Alice A. Hardigree

In an earlier report[5] we described some of the properties of an *Escherichia coli* K-12 mutant that failed to become resistant to ionizing radiation when grown in a glucose broth medium. Preliminary genetic information obtained from crosses between the mutant (AB1899) and a high frequency donor strain (HfrH) indicated that the gene responsible for this failure to show the "glucose effect" was located in the Lactose-T_6 region of the *E. coli* linkage map. More complete genetic analysis confirms this locus. This region comprises approximately 2–3% of the entire *E. coli* K-12 genome. By determining survival curves for cultures of different ages it has been possible to establish that the mutant allele at this locus also prevents the development of radiation resistance characteristically observed for cultures of *E. coli* passing from lag into log phase.[6] Microscopic studies of the growth and division of these mutant cells by a technique previously described[5] have established that many irradiated cells form long filaments that do not contain cross-septa. The frequency of these structures corresponds very well to the fraction of cells failing to form macrocolonies, as judged by plating techniques. Recently, several compounds have been found to reverse filament formation in *Erwinia*.[7] Pantoyl lactone is one of the most effective and we have incorporated it in the postirradiation plating medium. Figure 21 shows the effect of 0.08 *M* pantoyl lactone in a nutrient agar–yeast extract

[5]H. I. Adler and Alice A. Hardigree, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 71.

[6]G. E. Stapleton, *J. Bacteriol.* **70**, 357 (1955).

[7]E. A. Grula and M. M. Grula, *J. Bacteriol.* **83**, 981 (1962).

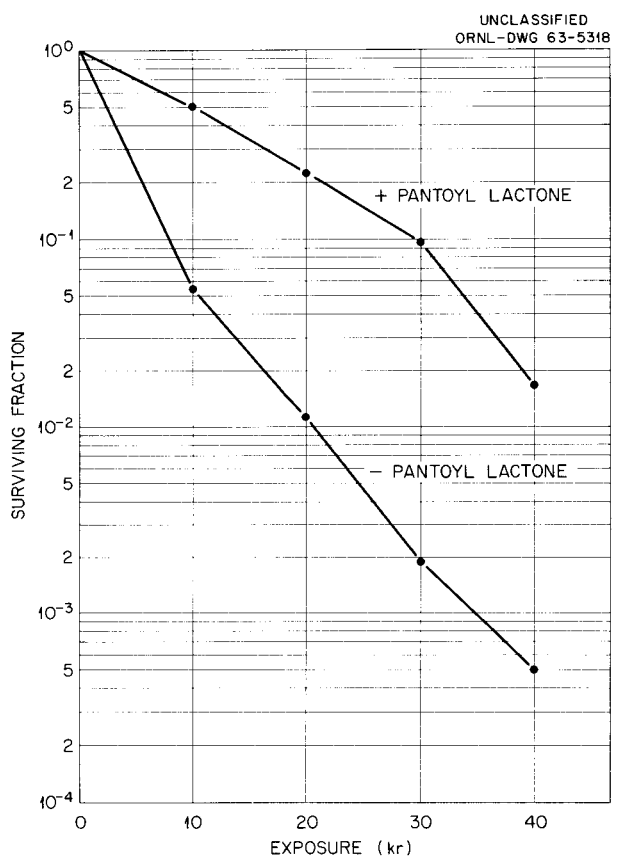

Fig. 21. Effect of Pantoyl Lactone in the Postirradiation Plating Medium on the X-Ray Sensitivity of a Culture of *E. coli* AB 1899 Grown in Glucose Broth.

medium. The data strongly suggest that the mechanism for cross-septum formation is unusually radiation-sensitive in *E. coli* AB1899 and that inactivation is due not to failure of cell growth but to the failure of septum formation and consequent cell division. Pantoyl lactone allows irradiated cells to form cross-septa and then divide to give rise to macrocolonies. We intend to investigate in greater detail the function of this genetic locus, since it seems to be involved in a basic aspect of cell division in *E. coli* and to have important consequences for sensitivity to both ionizing and ultraviolet radiation.

ATTEMPTS TO ISOLATE RAPIDLY DIVIDING MUTANTS OF *ESCHERICHIA COLI* AFTER EXPOSURE TO CARCINOGENIC AGENTS

K. A. Fry H. I. Adler

The carcinogenic agents, methyl-2-dichloroethylamine and *t*-butyl hydroperoxide, were spotted, in appropriate dilutions, at the center of Difco nutrient agar petri dishes, and an overlay of soft agar seeded with *Escherichia coli* K-12 HfrH was prepared. In some experiments, the carcinogens were first applied to small filter paper disks, which were then placed on the agar surface. When the methyl-2-dichloroethylamine was used, zones in which bacterial growth was completely or partially inhibited were observed. In one experiment, three colonies developed within a region of otherwise complete inhibition. These colonies were recharacterized and found to be identical to the parent culture with regard to streptomycin sensitivity, ability to act as genetic donors, growth rate, and sensitivity to the methyl-2-dichloroethylamine. They are, therefore, not mutants of the desired type. *t*-Butyl hydroperoxide displayed no bacteriostatic or bactericidal effect in our test system at any concentration, possibly owing to its limited water solubility. The methods of screening developed will be applied to other water-soluble carcinogens as they are made available.

AN *ESCHERICHIA COLI* K-12 Hfr STRAIN WITH THE FERTILITY FACTOR ATTACHED TO OR WITHIN THE STRUCTURAL GENE FOR β-GALACTOSIDASE

Roy Curtiss III

Introduction. – Hfr strains are donors which transfer their chromosome to F^- recipient strains at high frequency; F^+ strains are donors which transfer their chromosome at low frequency. In F^+ bacteria the fertility factor, F, replicates cytoplasmically in asynchrony with the bacterial chromosome and is transferred at high frequency to F^- recipient bacteria. In Hfr bacteria, F is attached to the chromosome, replicates in synchrony with the chromosome and is transferred to F^- recipients at very low frequency. Any one Hfr strain transfers its chromosome in a linearly oriented manner which is determined by the site of F attachment. The attached F is the last marker to be transferred. The leading end of the chromosome which is transferred first is called the origin (O). During the isolation of several lac^- mutants from an *E. coli* K-12 F^+ prototroph by ultraviolet irradiation, one lac^- isolate was obtained which had all the properties of an Hfr strain. This study was begun to determine whether the mutation to lac^- and the attachment of F were due to the same event or to independent events.

Results. – As a reference, the linkage relationships of the markers used in the experiments are as follows:

$$leu \ldots pro_1\ lac_y\ lac_z\ lac_o\ lac_i\ pro_2 \ldots ade_3 .$$

The distance from pro_1 to pro_2 is about 1.5% of the total linkage map. The entire lac_y to pro_2 segment can be transduced by one P1kc transducing phage.[8] Mutants in the pro_1^- group are all blocked in the first step(s) in proline biosynthesis, and mutants in the pro_2^- group are all blocked in the last step. All pro_2 mutants crossfeed pro_1 mutants. Mutants in the lac_y group are unable to synthesize normal β-galactoside permease, and mutants in the lac_z group are unable to synthesize normal β-galactosidase.[9] The lac_o mutations are

[8] N. M. Schwartz, *Genetics* **48**, 1357 (1963).

[9] F. Jacob and J. Monod, *J. Mol. Biol.* **3**, 318 (1961).

all clustered at the right end of the lac_z gene and, if in the O° state, prevent the synthesis of both β-galactoside permease and β-galactosidase.[9] The lac_i gene determines whether the synthesis of β-galactoside permease and β-galactosidase are constitutive (lac_i^-) or inducible (lac_i^+).[9]

Attempts to obtain lac^+ revertants in the Hfr lac^- strain (Hfr OR1) were unsuccessful. Removal of the lac^- trait by transduction with P1kc resulted in lac^+ strains which were F^-, as deduced from mating experiments. These lac^+ transductants were also resistant to the donor-specific RNA phage, R17, which had been grown on Hfr OR1. F^- strains are completely resistant to R17. Since the attached fertility factor can be transduced along with a neighboring structural gene,[10] and since the *lac* and pro_2 region can be jointly transduced,[8] it was decided to use P1kc grown on Hfr OR1 to transduce a lac^+ pro_2^- F^- strain. One out of 20 pro^+ transductants tested was an Hfr by mating tests, was sensitive to R17, and was lac^-. All these results strongly support the idea that the lac^- phenotype in Hfr OR1 was caused by attachment of F at some site within the *lac* region.

Preliminary time of entry experiments indicated that Hfr OR1 transferred its chromosome in the order: O lac_z lac_y pro_1 ... *leu* ... ade_3 ... pro_2 lac_i F. In these crosses the lac_z^+ gene is first transferred in 3 to 4 min. In crosses between Hfr OR1 and various lac_y^- leu^- and lac_z^- leu^- F^- strains, leu^+ lac^+ recombinants were obtained in all but one. The one F^- which did not yield lac^+ leu^+ recombinants has a lac^- mutation which is a deletion of two-thirds of the lac_z gene and of the lac_o region of the lac_z gene.[8] The ratio of the numbers of lac^+ leu^+ recombinants to leu^+ recombinants declined the closer the lac^- marker was to the origin of the Hfr OR1 chromosome. This is plausible, since recombination must occur between the origin and the positive allele of the first selected marker to get integration of that allele. As this distance becomes shorter and shorter, the probability of recombination within this distance becomes less and less.

Hfr OR1 produces no β-galactosidase with or without induction.[11] To prove that the lac_i^+ (inducible) marker is the last transferred by Hfr OR1 two types of experiments were done. In the first, Hfr OR1 (lac_z^- lac_i^+) was crossed with an F^- strain which was leu^- lac_z^- lac_i^-, and leu^+ lac^+ recombinants were selected and then tested for inducible or constitutive synthesis of β-galactosidase. If the lac_i^+ gene from Hfr OR1 was not transferred, then all of these leu^+ lac^+ recombinants should produce β-galactosidase constitutively. This was found to be the case. The second proof that the lac_i^+ gene was the last transferred by Hfr OR1 made use of a strain derived from Hfr OR1 which contained an F pro_2^+ plasmid. In any Hfr bacterium, the attached F can rarely become unattached and, in doing so, frequently takes a portion of the terminal end of the chromosome with it.[12] These plasmids replicate autonomously in the cytoplasm and are transferred in total with very high efficiency.[12] The F pro_2^+ plasmid was isolated in a cross with an F^- pro_2^- strain by selecting for pro_2^+ recombinants formed before the normal transfer time for pro_2^+ from Hfr OR1 (120 min). This F pro_2^+ plasmid was then transferred to an F^- lac_z^+ lac_i^- pro_2^- strain (constitutive) by selecting for pro_2^+ recombinants. One of these recombinants was tested for inducible or constitutive synthesis of β-galactosidase. If the F pro_2^+ plasmid contained the lac_i^+ gene, then β-galactosidase synthesis should occur only after induction. This is because the inducible state (lac_i^+) is dominant to the constitutive state (lac_i^-).[9] This recombinant had to be induced before β-galactosidase could be detected, and therefore, was of the genotype lac_z^+ lac_i^- pro_2^-/F lac_i^+ pro_2^+.

Discussion. – The results strongly indicate that F has attached to the lac_o region of the lac_z gene in Hfr OR1. For this reason it is predicted that Hfr OR1 can not synthesize any β-galactoside permease as well as any β-galactosidase. This prediction will be tested.

It may be possible to use Hfr OR1 and strains isolated from it to investigate the nature of F attachment. That is, when F "attaches" is it linearly integrated into the chromosome or is it really attached like a prophage? Also, it may be possible to study specific functions of F, for example genes in F which are responsible for specific pair formation in the process of conjugation.

[10] S. K. De Witt and E. A. Adelburg, *J. Bacteriol.* **83**, 673 (1962).

[11] The author thanks Dr. K. A. Fry for performing the β-galactosidase assays.

[12] F. Jacob and E. A. Adelburg, *Compt. rend.* **249**, 189 (1959).

METHOD FOR ISOLATION OF PARTIAL DIPLOID STRAINS IN *ESCHERICHIA COLI* K-12

Roy Curtiss III

Introduction. – Stable partial diploid strains would be useful for studies on dominance, gene control, gene mapping, and the mechanism of genetic recombination. Partial diploid strains are arbitrarily called stable if they undergo haploidization in a nonselective medium at rates of less than 1% per bacterium per generation. Ordinarily unstable and stable partial diploids are found at frequencies of 0.1% among any selected class of recombinants in donor times recipient crosses.[13] The present study was begun to determine methods of increasing the yield of stable partial diploid strains.

Results. – In crosses using different donor strains and the same F^- recipient, in which the same recombinant classes were selected, the frequency of partial diploids was found to vary from less than 0.1% for HfrH to about 5% for $\chi 15(F^+)$ and Hfr OR1. To obtain stable partial diploid strains, it was necessary to have a pleiotropic deletion mutation (i.e., $/3,7,\lambda,pro_1^-$ or $/1,try^-$) in the F^- recipient strain. Only unstable partial diploid strains were found among pro^+ recombinants when the F^- recipient had a pro_1^- point mutation. (Unstable partial diploid strains are those which undergo haploidization at rates of 5–10% per bacterium per generation.) The yield of partial diploid strains was increased 2- to 4-fold when the F^- recipient had a $/3,7,\lambda,pro_1^-$ mutation instead of a pro_1^- point mutation. The selection of try^+ recombinants in a cross between a $/1,try^-$ mutant of $\chi 15$ and an F^- with a very closely linked anthranilic acid dependence mutation, yielded partial diploid strains at a frequency of about 30% in each of three experiments. Attempts to isolate similar yields of partial diploids by selection of pro^+ lac^+ recombinants in crosses between F^+ or Hfr donors with the markers pro^+, lac_y^-, lac_z^+, and an F^- with the markers $/3,7,\lambda,pro_1^-$, lac_y^+, and lac_z^- were unsuccessful. Only one partial diploid strain which is diploid for the regions encompassing the pro_1 and *lac* loci has been isolated. This result is surprising, since there is less than 0.1% recombination between the $/3,7,\lambda,pro_1^-$ and lac_y^- loci. Fifty percent of all partial diploids which were isolated as pro^+ recombinants in crosses with an F^- having the $/3,7,\lambda,pro_1^-$ marker were diploid for the *ara* to pro_1 region of the chromosome (9% of the genome). No similar analysis can be made for the partial diploids in the *try* region because of the paucity of genetic markers. All partial diploid strains obtained from crosses with the Hfr donors and 50% of those obtained in F^+ crosses were F^-.

Discussion. – In crosses employing HfrC (0 T6 *lac* pro_1 *ara* ... *try gal sfa*) and Hfr OR1 (0 lac_y pro_1 *gal* T6 pro_2 *sfa*) the isolation of stable partial diploids for the pro_1 to *ara* region and for the *try* region necessitated the derivation of the extra chromosomal fragment (exogenote) from the center of the Hfr chromosome. Presumably the fragmentation of the donor chromosome occurred after chromosome transfer, with consequent loss of all but the exogenote by postzygotic elimination. Attempts are now being made to prove or disprove this hypothesis.

Partial diploid strains isolated among pro^+ recombinants in crosses with F^- strains with $/3,7,\lambda,pro_1^-$ mutations had exogenotes which extended past pro_1 but not to lac_y (all but one) and past *ara* but not to *thr* (50%). This finding, which was independent of the strains used, indicates that there are nonrandom "breakpoints" in the bacterial chromosome. It is important to note that the region between the pro_1 and lac_y loci has been associated with breakpoints in all of three transpositions observed in *E. coli* K-12,[14,15] with a site for F attachment[14] and with a site of a chromosome aberration, making the chromosome linear.[16]

[13] Joshua Lederberg *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **16**, 413 (1951).

[14] F. Jacob and E. L. Wollman, *Sexuality and the Genetics of Bacteria*, Academic Press, New York, 1961.

[15] S. K. De Witt and E. A. Adelburg, *Genetics* **47**, 577 (1962).

[16] Roy Curtiss III, manuscript in preparation.

UV-INDUCED GENETIC RECOMBINATION IN A PARTIAL DIPLOID STRAIN OF *ESCHERICHIA COLI* K-12

Roy Curtiss III

The process of ultraviolet light (uv)-induced genetic recombination has been studied using a partial diploid strain of *E. coli* K-12. This strain is diploid from the locus controlling arabinose utilization (*ara*) to the locus controlling the first step(s) in proline biosynthesis (pro_1). The extra chromosomal fragment (exogenote) contains the markers ara^+, leu^+, $T1^S$, and pro_1^+. The endogenote, which is the region of the chromosome complemented by the exogenote, contains the markers ara^-, leu^-, $T1^R$, and $/3,7,\lambda,pro_1^-$. The last mutation is pleiotropic and confers resistance to the phages T3, T7, and λ and a requirement for proline. This partial diploid strain spontaneously undergoes haploidization at a rate of 0.4% per bacterium per generation in nonselective medium and recombination with retention of the diploid state, which leads to homozygosity at one or more loci, at a rate of 0.02% per bacterium per generation.[17] This strain divides by the formation of uninucleate cells from binucleate cells.

Recent work on uv-induced recombination in this strain has confirmed earlier findings.[18] These can be briefly summarized as follows: Both uv-induced haploidization and uv-induced recombination with retention of the diploid state occur by a conservative mechanism. In other words, clones containing only recombinant cells are obtained by plating on nonselective medium immediately after uv irradiation. Recombination is obligatory to obtain uv-induced haploidization. This is also true for spontaneous haploidization.[17] Recombination with retention of the diploid state is nonreciprocal. Analysis of the frequencies with which different recombinant types are formed has led to the conclusion that the *ara* to pro_1 region of the chromosome is replicated (duplicated) late in the chromosome replication cycle.

Presently being investigated are photoreactivation of uv-induced recombination, the dose-response curve for uv-induced recombination, uv-induced recombination in stationary phase cultures, and the occurrence of reciprocal recombination. Preliminary studies have shown that colony-forming ability is much more efficiently photoreactivated than is uv-induced recombination. The frequency of any recombinant type among survivors increases linearly with uv dose up to a dose of 600 ergs/mm^2. The total frequency of recombinants among survivors levels off at 70–80% at a dose of 1200 ergs/mm^2 or above. The maximum number of recombinants, based on the cell concentration of the unirradiated control, is obtained at a dose of about 300 ergs/mm^2 (30% survival). The frequencies of uv-induced recombination in stationary phase cells indicate that the chromosomes are all duplicated and/or all the cells are binucleate. Special sensitive procedures have been developed to detect reciprocal recombination in this partial diploid strain. At present, no recombinants with reciprocal exchanges have been found.

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[17] Roy Curtiss III, manuscript in preparation.

[18] Roy Curtiss III, *Bacteriol. Proc.* p 29 (1963).

Mammalian Recovery

Secondary Disease		Recovery *in Vitro*	
C. C Congdon	Frances E. Hacker	L. H. Smith	T. W. McKinley, Jr.
W. H. McArthur[a]	Kathleen H. Harrington		Karen T. Bredahl[f]
Stanfield Rogers[a]	Alberta P. Henley		
Vu-Thi-Suu[b]	M. G. Hanna, Jr.[d]		
D. C. Swartzendruber[c]	Joseph Kendrick[e]	**Hemopoietic and Lymphatic Stem Cells**	
	Marjorie A. Stewart[f]	Joan Wright Goodman	Wanda C. Shelton
			Joan Underwood

[a]Consultant.

[b]Visiting investigator from the Dalat Institute of Applied Nuclear Research, Viet Nam, sponsored by the Agency for International Development.

[c]Research associate.

[d]ORINS Fellow.

[e]Temporary summer employee.

[f]Student trainee.

LYSOZYME ACTIVITY IN THE KIDNEY AND OTHER ORGANS OF IRRADIATION CHIMERAS

Vu-Thi-Suu

Introduction. – It was shown in an earlier report that a marked increase occurs in lysozyme activity in the kidneys of irradiated mice treated with homologous bone marrow (HBM).

To determine the source of increased kidney lysozyme, the investigation was repeated, with a more complete time course on the kidney measurement, and the lysozyme activity in different organs of irradiation chimeras was determined.

Methods and Results. – Male mice ($1C3F_1$/Cum), 12 to 18 weeks of age, were used as recipients or donors of isologous bone marrow (IBM). For the donors of HBM, male mice ($B6D2F_1$/Cum), 12 to 29 weeks of age, were used. Bone marrow cells (40×10^6) were given to the animals within 4 hr after irradiation.

The animals were weighed and then decapitated. The tissues were removed, weighed, and homogenized 5 min with $^1/_{15}$ *M* phosphate buffer (pH 7.3). The mixture was then centrifuged 30 min at 14,000 rpm in a refrigerated centrifuge. The supernatant was used for the lysozyme assay, and the sediment was discarded.

An enzyme activity measurement was made with a Zeiss spectrophotometer (wavelength, 45 mμ). The temperature was controlled at 25°C. The procedure of Shugar[1] standardized against Worthington lysozyme was used in the lysozyme assay. Absorbance was read at 30-sec intervals, and the course of the reaction was recorded for $2^1/_2$ min.

Figure 22 is a plot of lysozyme activity (referred to a standard enzyme preparation) in kidney homogenates of normal mice, x-irradiated mice (950 r), x-irradiated mice treated with IBM, and

[1]D. Shugar, *Biochim. Biophys. Acta* **8**, 302 (1952).

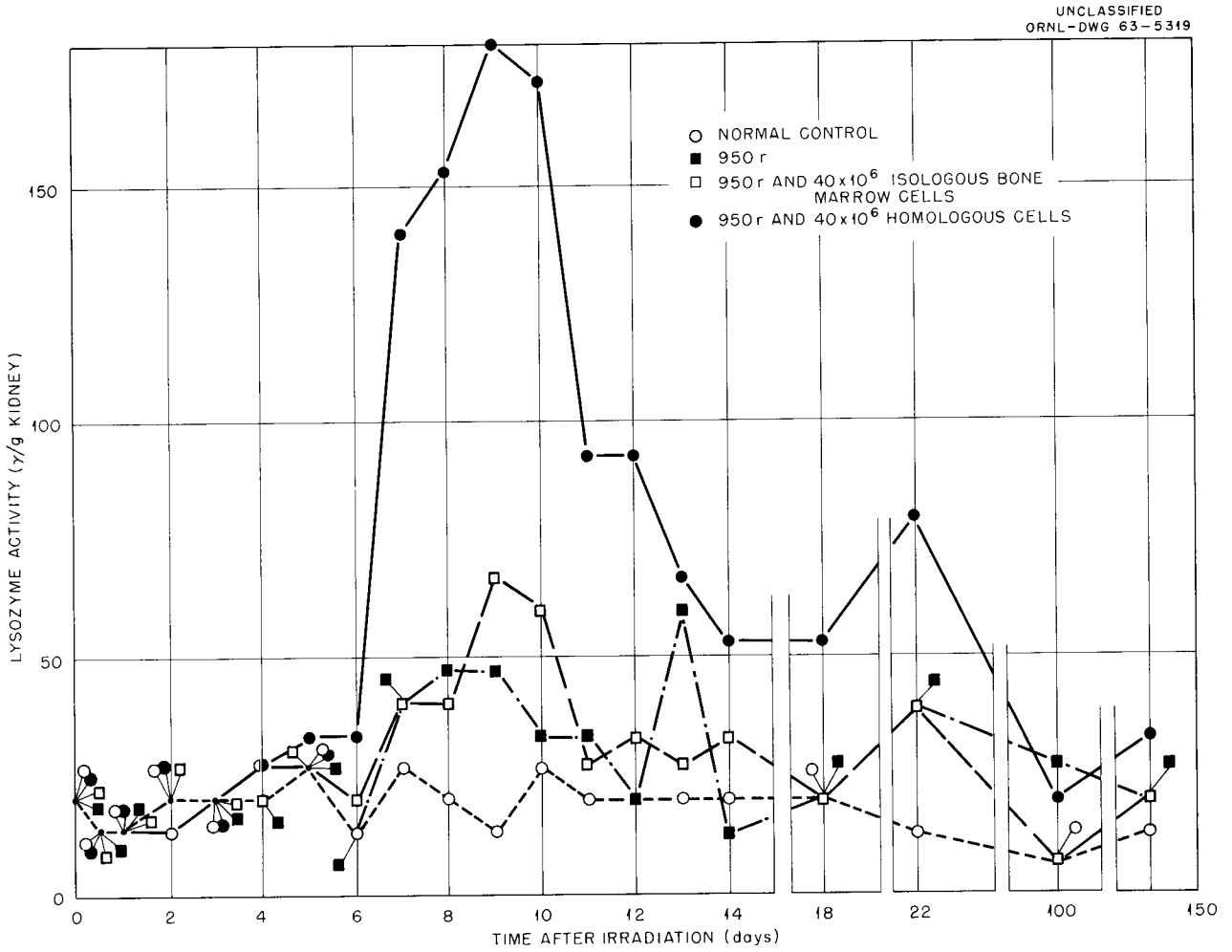

Fig. 22. Lysozyme Activity in Kidneys of Mice. Each point is the average of values from three mice per group.

x-irradiated mice treated with HBM. In the homologous chimeras, during the first six days after irradiation and treatment with bone marrow, the lysozyme is almost at the normal value; but at day 7 there is a marked increase which reaches a peak at nine days and then rapidly decreases and is only slightly above the normal value during the third week. Data from kidney homogenates of homologous chimeras at 100 and 150 days after irradiation and bone marrow treatment show a normal value of lysozyme activity as it had been presumed in a previous work.[2] There is a slight increase in lysozyme activity in the irradiated mice and in mice irradiated and treated with IBM during the second week.

Table 18 shows the lysozyme content in different organs of mice killed at nine days after receiving irradiation and bone marrow treatment. Normal and x-irradiated mice (950 r) were killed along with the irradiated and bone-marrow-treated animals as control groups.

Lysozyme assay of the different organs of normal mice indicated highest values in bone marrow, small intestine, lung, kidney, spleen, and colon. The small intestine, lung, and kidney did not show the marked decrease found in bone marrow, spleen, and colon at nine days after irradiation.

[2]Vu-Thi-Suu, C. C Congdon, and A. L. Kretchmar, *Proc. Soc. Exptl. Biol. Med.* 113, 481 (1963).

Table 18. Lysozyme Activity in Different Organs of Normal Mice, of Mice Nine Days After Irradiation (950 r Total-Body Irradiation), of Mice Irradiated and Treated with 40×10^6 IBM or HBM Cells[a]

Tissue	Lysozyme Activity (γ/g of Tissue)			
	Normal Control	X Ray Control	IBM	HBM
Bone marrow	180 (11)	29 (10)	350 (13)	459 (13)
Small intestine	51 (3)	81 (3)	57 (3)	53 (3)
Lung	48 (4)	36 (3)	56 (4)	64 (3)
Kidney	28 (3)	21 (3)	42 (3)	196 (3)
Spleen	17.3 (5)	3.8 (13)	39 (4)	56 (3)
Colon	17 (3)	4 (6)	6 (3)	4 (4)
Lymph node	8 (5)	0 (11)	22 (8)	25 (3)
Submaxillary gland	4.6 (5)	5.2 (5)	2.6 (5)	2.4 (5)
Thymus	3.4 (11)	0 (34)	3.8 (22)	6.6 (23)
Liver	2.2 (3)	4.4 (3)	4.0 (3)	6.6 (3)
Plasma	1 (3)	1 (7)	0 (4)	1 (3)

[a]The number of mice is given in parentheses.

In the irradiated chimeras at nine days, bone marrow, kidney, spleen, and lymph node showed marked increase, whereas the other organs changed relatively little. The colon showed a persistent decrease, as in the colon of the x-ray control.

The biological significance of lysozyme and the meaning of the changes found in radiation chimeras are not known. The greatest increase in activity at nine days was in the bone marrow of the HBM mice. Presumably, this was the source of the elevated kidney lysozyme in these mice since the assumption has been that the kidney only filters out the lysozyme rather than produces it. Further work will be needed to understand the lysozyme activity changes and its role in secondary disease.

EFFECT OF RETICULOENDOTHELIAL STIMULATION ON BONE MARROW TRANSPLANTATION

Marjorie A. Stewart

Introduction. – It has been demonstrated that stimulation of the reticuloendothelial system (RES) before x irradiation and transplantation of bone marrow influences the survival time of the host. According to Wooles and Di Luzio,[3] stimulation of the RES by glucan reduces 30-day survival of irradiated mice injected with foreign bone marrow, presumably by killing the transfused cells. They did not observe, however, an effect of this agent on transplantation of isologous bone marrow (IBM).

The present work was an attempt to obtain additional data on reticuloendothelial hyperfunction and bone marrow transplantation with the employment of various substances known to be RES stimulants.

Methods. – The stimulants tested were India ink, estrogen, and glucan. Male and female $(C57BL \times A)F_1$ or $(101 \times C3H)F_1$ mice, 10 to 24 weeks old, were used. One group received 1 ml of a 1:10 dilution of India ink intravenously two days before x irradiation. A second group was given 1 ml daily of the same ink solution intravenously for three days prior to x irradiation.

[3]W. R. Wooles and N. R. Di Luzio, *Am. J. Physiol.* **203**, 404 (1962).

Estrogen (1 mg) was administered intraperitoneally three days prior to x irradiation. Glucan[4] was given intravenously (4 mg per 100 g of body weight) daily three days prior to x irradiation. Doses of 40×10^6 IBM and homologous bone marrow (HBM) cells were used intravenously; with rat bone marrow the dose was 100×10^6.

Results and Conclusions. – No significant effect of India ink, estrogen, or glucan (Table 19) was observed on survival time in mice injected with either IBM or HBM. The failure of these agents to show an adverse effect on HBM transplantation by reticuloendothelial hyperfunction can be explained in part, perhaps, by the fact that different mouse strains were used and higher irradiation dosage was given in this work. On the other hand, the data (Table 19) suggest that in the glucan-treated mice, interference of rat bone marrow transplantation occurred, as judged by mean death time. This observation substantiates that of Wooles and Di Luzio[3] with heterologous marrow and seems to indicate, as Wooles and Di Luzio suggest, that as a result of reticuloendothelial hyperfunction, injected cells may be phagocytized or antibody formation stimulated, thereby hampering bone marrow transplantation.

[4]Kindly supplied by N. R. Di Luzio, Department of Physiology, University of Tennessee Medical Units, Memphis, Tennessee.

Table 19. Effect of Glucan on Transplantation of Bone Marrow in Lethally Irradiated Mice

Recipient	Donor	Treatment	Total Mice	Number of Deaths/Total Mice		
				7 Days	14 Days	21 Days
$(C57BL \times A)F_1$		950 r only	5	2/5	4/5	5/5
$(C57BL \times A)F_1$		Glucan + 950 r	4	0/4	1/4	2/4
$(C57BL \times A)F_1$	$(C57BL \times A)F_1$	950 r + IBM	6	1/6	1/6	1/6
$(C57BL \times A)F_1$	$(C57BL \times A)F_1$	Glucan + 950 r + IBM	5	0/5	0/5	0/5
$(C57BL \times A)F_1$	$(101 \times C3H)F_1$	950 r + HBM	4	1/4	1/4	1/4
$(C57BL \times A)F_1$	$(101 \times C3H)F_1$	Glucan + 950 r + HBM	5	1/5	1/5	2/5
$(C57BL \times A)F_1$, $(101 \times C3H)F_1$		950 r only	20	11/20	19/20	19/20
$(C57BL \times A)F_1$, $(101 \times C3H)F_1$		Glucan + 950 r	10	4/10	10/10	10/10
$(C57BL \times A)F_1$, $(101 \times C3H)F_1$	Rat	950 r + rat bone marrow	24	10/24	18/24	18/24
$(C57BL \times A)F_1$, $(101 \times C3H)F_1$	Rat	Glucan + 950 r + rat bone marrow	33	29/33	30/33	30/33

MORTALITY FROM SECONDARY DISEASE IN RADIATION CHIMERAS

C. C Congdon

It was first recognized about 1954 that a secondary disease process developed in irradiated mice treated with foreign bone marrow. The presence of secondary disease severely limited the value of foreign marrow therapy since a very high mortality usually results from the syndrome.

For practical purposes, all efforts to circumvent secondary disease have failed; however, some specific variables, such as younger ages of donor tissue, are associated with a reduced severity of the disease. The most intriguing finding seen with foreign marrow transplantation is the spontaneous circumvention of secondary disease or the spontaneous cure, yet all attempts to understand the factors responsible for these spontaneous changes favoring survival of the foreign marrow chimera have been unsuccessful.

The search for biochemical or metabolic parameters that might vary from experiment to experiment or animal to animal is one major approach being undertaken.[5]

A second approach has been started in collaboration with M. A. Kastenbaum and D. A. Gardiner of the ORNL Mathematics Division. This approach has two facets: One is a retrospective study of all foreign bone marrow experiments carried out in a single laboratory of the Biology Division since 1955. Data from these experiments were placed on IBM cards for use with mechanical data-processing techniques. The plan is to reexamine 90-day cumulative mortality in all rat and homologous bone marrow chimeras with a sorting and combining of data for all variables involved including strain of animal, sex, age of donor, amount of cells transplanted, day of transplantation, etc. From this material a search for influential factors in spontaneous recovery from secondary disease will be made.

[5]Vu-Thi-Suu and W. H. McArthur, this report.

Table 20. Experimental Design for Study of Selected Variables in Mortality from Secondary Disease in Homologous Bone Marrow Transplantation[a]

Group	Bone Marrow Cell Dose	Day of Injection[b]	Age of Donor Cells (Day in Relation to Birth)[c]	Sex
	($\times 10^6$)			
1	50	3	90	M
2	50	3	−1 to +2	F
3	50	0	90	F
4	50	0	−1 to +2	M
5	10	3	90	F
6	10	3	−1 to +2	M
7	10	0	90	M
8	10	0	−1 to +2	F
9	30	1½	45	M
10	30	1½	45	F

[a]The recipient mice ($1C3F_1$, three months old) received 950 r.

[b]Refers to time after x irradiation.

[c]Day −1 to +2 means liver taken one day before birth to two days after birth.

The other facet is a prospective study designed by Gardiner and Kastenbaum to determine the possible interaction of variables that have been known, or suspected, in the past to have an influence on the mortality from secondary disease. These selected variables are bone marrow dose, day of injection, age of donor, and sex.

Table 20 gives the experimental design. Fetal liver and newborn liver cells are used as the source of the youngest "bone marrow" cells. Considerable difficulty was encountered in evaluating the number of these cells because of the debris present in the cell suspensions. Two weeks were required to carry out the experimental design a single time.

Table 21 shows the partial results of three replicate experiments. So much early mortality from radiation was obtained using fetal and newborn liver as a source of "bone marrow" cells that some modification of design will probably be necessary. Otherwise, the usual patterns of mortality from secondary disease seem to be occurring in the experiments.

Further results from the retrospective and prospective studies will be given in subsequent progress reports.

Table 21. Preliminary Data Obtained from Use of Experimental Design Shown in Table 20

Group	BM Dose	Day of Injection	Age of Donor (days)	Sex	Number of Mice	Total Number of Mice Dead by Day										
						5	10	15	20	25	30	35	40	45	50	55
	($\times 10^6$)															
1	50	3	90	M	15	0	0	0	0	1	3	6	6	7	7	8
2	50	3	−1 to +2	F	15	0	6	7	7	7	7	7	7	7	7	7
3	50	0	90	F	15	0	0	0	0	0	2	10	12	12	12	12
4	50	0	−1 to +2	M	15	1	1	4	6	8	9	10	10	10	10	10
5	10	3	90	F	15	0	0	0	0	2	4	5	7	7	7	7
6	10	3	−1 to +2	M	15	0	10	13	13	13	13	14	14	15		
7	10	0	90	M	11	0	0	0	1	2	3	4	4	4	5	5
8	10	0	−1 to +2	F	14	0	9	14								
9	30	1½	45	M	15	0	0	1	1	1	1	1	4	7	8	8
10	30	1½	45	F	15	0	0	0	0	0	0	2	2	3	4	4
1	50	3	90	M	14	0	0	0	0	0	1					
2	50	3	−1 to +2	F	15	0	5	8	8	9	9					
3	50	0	90	F	15	0	0	0	2	3	4					
4	50	0	−1 to +2	M	15	0	0	1	1	1	2					
5	10	3	90	F	15	1	1	1	1	1	1					
6	10	3	−1 to +2	M	15	0	3	11	13	13	13					
7	10	0	90	M	15	0	0	2	2	2	2					
8	10	0	−1 to +2	F	15	0	3	13	14	15						
9	30	1½	45	M	15	0	0	0	0	0	3					
10	30	1½	45	F	15	0	0	0	0	0	0					
1	Not done		Not done													
2	50	3	−1 to +2	F	15	0	4	5	5	5	5					
3	50	0	90	F	15	0	0	0	0	0	0					
4	50	0	−1 to +2	M	15	0	0	0	2	3	3					
5	10	3	90	F	15	0	0	0	0	2	2					
6	10	3	−1 to +2	M	15	0	5	6	9	9	9					
7	10	0	90	M	15	0	0	1	2	2	2					
8	10	0	−1 to +2	F	15	0	6	14	14	14	14					
9	30	1½	45	M	15	0	0	0	0	0	0					
10	30	1½	45	F	15	0	0	0	0	0	0					

OXYGEN CONSUMPTION IN LETHALLY IRRADIATED MICE GIVEN BONE MARROW INTRAVENOUSLY

W. H. McArthur

Introduction. – Whole-animal-metabolism studies following foreign bone marrow transplantation are few. McRae[6,7] measured food intake, body weight changes, and reticuloendothelial activity.

Preliminary studies by McArthur *et al.*[8] showed that mice given foreign bone marrow after lethal irradiation stay in positive nitrogen balance for 60 days, even in the presence of a small weight loss. A very mild clinical secondary disease was present in these animals.

As a continuation of whole-animal-metabolism studies in radiation chimeras, the present report summarizes oxygen consumption in lethally irradiated mice given foreign, homologous bone marrow. The measurements were made over a period of 90 days after irradiation and marrow transplantation. Measurements on normal mice and those receiving isologous bone marrow extended through the 44th week.

Methods and Results. – All the oxygen consumption measurements were made on LAF_1 male mice individually caged. The animals were 12 to 15 weeks of age at the start of the experiment. Food and water were always available except during the measurement period.

Three groups of animals were studied. One group was a normal control, and two groups received 900 r total-body x irradiation, followed within 30 min by an intravenous injection of 40×10^6 bone marrow cells of isologous (IBM) or homologous $(101 \times C3H)F_1$ (HBM) origin.

Ten normal LAF_1 mice, 7 IBM, and 17 HBM animals were used in the oxygen consumption study. No deaths occurred in the normal and the IBM animals during the experiment. In the HBM group, 14 of the 17 mice died on the following days: 4, 6, 11, 13, 13, 16, 27, 28, 32, 36, 61, 61, 70, and 107.

Tables 22 and 23 show the time intervals at which oxygen consumption measurements were made for each group as well as weekly averages. Measurements were not made on HBM mice after the 13th week, whereas some measurements were made on normal and IBM mice through the 44th week.

[6] James McRae, personal communication.

[7] James McRae, Ph.D. thesis, University of California, Berkeley (1960).

[8] W. H. McArthur, A. Kretchmar, and C. C Congdon (abstract), *Blood*, in press

Table 22. Oxygen Consumption Measurements (1 to 13 Weeks After Irradiation)

Time (weeks)	Normal		IBM		HBM		Average O_2 Consumed ($cm^3\ min^{-1}\ g^{-1}\ week^{-1}$)		
	Number/Day	Number/Week	Number/Day	Number/Week	Number/Day	Number/Week	Normal	IBM	HBM
1	1–10	30	3–6	30	4–12	48	1.73	1.45	1.46
2	1–4	12	1–3	14	3–11	45	1.78	1.76	1.57
3	1–4	11	1–4	18	2–7	28	1.91	1.62	1.57
4	1–3	12	2–5	23	1–6	26	1.79	1.67	1.57
5	1–5	13	2–5	21	1–3	10	1.68	1.62	1.60
6	1–3	6	1–4	14	1	4	1.82	1.76	1.57
7	1–2	7	1–3	15	1	2	1.81	1.80	1.56
8	1–3	9	1–3	14	1–2	3	1.75	1.68	1.84
9	1–2	8	1–5	12	1	2	1.77	1.76	1.74
10	2–3	11	1–2	9	0	0	1.69	1.55	
11	1–3	6	1–3	11	1	1	1.86	1.78	1.69
12	1–3	10	1–3	12	1–3	9	1.86	1.80	1.75
13	2–3	11	1–3	11	1–2	4	1.82	1.89	1.71

Table 23. Oxygen Consumption Measurements (14 to 44 Weeks After Irradiation)

Time (weeks)	Normal		IBM		Average O_2 Consumed ($cm^3\ min^{-1}\ g^{-1}\ week^{-1}$)	
	Number/Day	Number/Week	Number/Day	Number/Week	Normal	IBM
14	1–4	12	1–4	10	1.96	1.83
15	0–3	12	1–4	11	1.91	1.81
16	1–4	16	1–2	11	2.13	1.86
17	2–4	17	1–2	8	1.91	1.88
18	2–4	13	1–2	11	1.87	1.90
19	1–2	4	1–3	6	1.99	1.86
20	1–2	6	0–2	8	1.97	1.88
21	0–1	3	1–2	5	1.87	1.88
22	0–1	1	0–1	3	1.92	1.78
23	0–1	1	0–1	3	1.86	1.77
24	0–1	2	0–1	3	1.97	1.85
25	0–1	2	1–2	3		
26	0	0	0	0		
27	0	0	0–1	1		
28	0–1	2	0–1	4	2.15	2.00
29	0–1	2	0–1	2	1.65	1.88
30	0–1	2	0–1	2	2.03	2.13
31	0–1	3	0–1	3	2.21	2.02
32	0–1	2	0–1	2	1.88	1.80
33	0–1	2	0–1	2	2.18	2.08
34	0	0	0	0		
35	0–1	2	0–1	2	1.92	1.99
36	0–1	2	0–1	2	1.88	1.86
37	0	0	0	0		
38	0–1	4	0–1	4	2.07	1.83
39	0–1	2	0–1	2	2.14	2.04
40	0–1	1	0	0	2.07	
41	0–1	1	0–1	1	2.20	1.89
42	0–1	3	0–1	1	2.13	1.55
43	0–1	1	0–1	1	2.14	2.06
44	0–1	2	0–1	1	2.29	1.80

It can be seen (Table 22) that IBM and HBM mice have a lower oxygen consumption than normal mice during the 13-week period of measurement after irradiation. HBM mice are nearly always lower than IBM mice.

From 14 to 44 weeks after x irradiation (Table 23), the IBM mice continue to consume less oxygen than the normal controls.

The significance of these findings is not entirely clear. Statistical evaluation of the results has not been completed.

It has been observed that HBM are less active than normal mice, and perhaps this accounts for the decrease in oxygen consumption.

IRRADIATION OF DONOR BONE MARROW: EFFECTS ON SURVIVAL OF IRRADIATED RECIPIENT MICE

Joan Wright Goodman

Introduction. – One method that has been used in an attempt to prevent or ameliorate secondary disease is irradiation of donor material. The rationale of this procedure rests on the assumption that immunologically competent cells responsible for secondary disease are more radiosensitive than myelopoietic cells. Although data suggestive of a preferential elimination of immunologically active cells by irradiation have appeared,[9,10] radiosensitivity measurements made directly on the two kinds of cells indicate that the D_{37} values are similar.[11–15] This report covers experiments designed to evaluate 90-day survival of irradiated mice treated with either unirradiated homologous marrow or homologous marrow irradiated *in vivo* before injection.

Methods. – Recipient mice were [101/Cum ♀ × C3H/Anf Cum ♂]F_1 (1C3F_1), and donors were either [C57L/Cum ♀ × A/He Cum♂]F_1 (LAF_1) or [C57BL/6 ♀ × DBA/2 Cum ♂]F_1 (B6D2F_1) male or female mice, three to five months old. Recipient mice, exposed to a single, whole-body dose of x rays (950 r), 24 hr before bone marrow injection, were the same sex as their donors in all cases. Irradiated donors were exposed to 400 r 30 min to 4 hr before their bone marrow cells (BMC) were taken and injected intravenously into the irradiated recipients. Irradiated BMC dose was set arbitrarily at 100×10^6, and the unirradiated dose was set at that number of cells estimated to survive out of 100×10^6 at 400 r (ref 11) (2.5×10^6).

Daily mortality checks were made, and after 90 days all survivors were tested for the presence of donor-type erythrocytes. Some mice were killed for necropsy and examination of lymph nodes for donor-type cells by a cytotoxicity test.

Results. – Survival results are summarized in Table 24, and presented in more detail in Table 25. It can readily be seen from the summarized data that irradiation of marrow did not give rise to increased 90-day survival and presumably, therefore, did not decrease secondary disease in

[9] G. Cudkowicz, *Proc. Soc. Exptl. Biol. Med.* **107**, 821 (1961).

[10] Bertie F. Argyris, *Transplantation Bull.* **29**, 10 (1962).

[11] E. A. McCulloch and J. E. Till, *Radiation Res.* **13**, 115 (1960).

[12] T. Makinodan, M. A. Kastenbaum, and W. J. Peterson, *J. Immunol.* **88**, 31 (1962).

[13] F. Celada and Rachel R. Carter, *J. Immunol.* **89**, 161 (1962).

[14] G. S. Hodgson, *Acta Physiol. Latinoam.* **12**, 365 (1962).

[15] L. H. Smith and O. Vos, *Radiation Res.* **19**, 485 (1963).

Table 24. Summary[a] of Survival Data from 1C3F_1 ♂ and ♀ Mice Irradiated with 950 r

Donor	Number of Mice	Inoculum (BMC)[b]	Surviving at 90 Days (%)
		($\times 10^6$)	
	48	None	0[c]
LAF_1	131	2.5	42.7
	94	100.2	43.6
	124	None	0[c]
B6D2F_1	94	2.5	37.2
	105	100.0	25.7

[a] Data pooled from seven experiments shown in detail in Table 25.

[b] The 2.5×10^6 values are for BMC from unirradiated donors; the 100.2×10^6 and 100.0×10^6 values are for BMC from donors exposed to 400 r *in vivo*.

[c] All controls dead by day 13 after irradiation.

Table 25. Survival Data from Irradiated Mice Given Irradiated or Unirradiated Homologous Bone Marrow

Experiment	Sex of Donor and Recipient	Inoculum (BMC)[a]	Number of 950 r $1C3F_1$ Recipients Alive Days After Irradiation				Surviving at 90 Days (%)
			0	30	60	90	
		($\times 10^6$)					
LaF_1 donors							
A	♂	2.5	55	44	24	21	38.2
	♂	100.0	38	26	15	9	23.7
B	♂	2.5	36	32	26	24	66.7
	♀	2.5	35	24	11	11	31.4
	♀	100.0	12	11	7	4	33.3
C	♂	2.5	5	3	0	0	0
	♂	100.0	9	9	6	5	55.6
	♀	100.0	35	32	27	23	65.7
$B6D2F_1$ donors							
D	♂	2.5	20	18	9	6	30.0
	♂	100.0	8	8	3	2	25.0
	♀	2.5	10	10	7	6	60.0
	♂	100.0	5	4	3	1	20.0
E	♂	2.5	35	29	12	9	25.7
	♂	100.0	15	15	9	6	40.0
	♀	100.0	8	7	0	0	0
F	♀	2.5	10	9	3	1	10.0
	♀	100.0	36	31	12	6	16.7
G	♀	2.5	9	8	5	5	55.6
	♀	1.0	16	13	4	3	18.8
	♀	0.5	23	13	6	4	17.4
	♀	100.0	4	4	2	1	25.0
	♂	2.5	10	10	8	8	80.0
	♂	1.0	20	16	9	6	30.0
	♂	0.5	30	11	6	4	13.3
	♂	100.0	29	26	15	11	37.9

[a]The 100.0×10^6 values are for BMC from donors exposed to 400 r *in vivo*; all other values are for BMC from unirradiated donors.

either of the two strain combinations tested. Of 172 radiation control mice (exposed to 950 r and untreated) representing all seven experiments, one died on day 5, two on day 6, and the rest on days 7 to 13 after irradiation.

When the 90-day survivors (79) were tested for chimerism, all had donor-type red cells, regardless of whether donor BMC had been irradiated or not. Lymph node cells from 19 chimeras (8 that had received unirradiated and 11 that had received irradiated BMC) were reacted with specific isoimmune, cytotoxic sera in parallel with cells from untreated control mice of donor and host type. In all cases, chimeric lymph node cells were identified as donor type.

The similarity of the two groups was also apparent at autopsy. Some nodes were atrophied, and some appeared to be well regenerated. The condition of lymphatic tissue could not be correlated with the preinjection treatment of donor BMC nor was it uniform throughout an individual chimera.

These data, showing that irradiation of homologous donor bone marrow does not decrease secondary disease, are in contrast to those of Cudkowicz[9] but are in complete accord with radiosensitivity measurements showing similar D_{37} values for hemopoietic and immunologically competent cells.[15] Inasmuch as there does not appear to be any ameliorative effect on secondary disease of irradiating donor cells, it seems unlikely that the technique will be seriously considered as a therapeutic measure.

EFFECT OF FETAL THYMUS TRANSPLANTATION ON MORTALITY FROM SECONDARY DISEASE

Kathleen H. Harrington

In an earlier report[16] the effect of fetal thymus injection on mortality from secondary disease was reported. The amount of thymus cells injected was in the range of 10 to 17 million. It was felt that a larger number of cells should be tried before the attempt to circumvent secondary disease with fetal thymus cells was discontinued. The results of a study with larger doses of cells are given in Table 26.

It can be seen that A·CA fetal thymus of the donor bone marrow type (A·CA-HBM) in the first experiment had no significant effect on mortality from secondary disease. The number of fetal thymus cells ranged from 27 to 68 million.

In the second experiment the foreign bone marrow (HBM), thymus, and recipient mice were all foreign to each other, and in this case also there was no effect on 90-day mortality, even though as many as 85×10^6 cells were injected.

These negative results show, however, that the fetal thymus lacks immunological competence since one would expect a greater mortality than in HBM alone if immunological competence were present in the thymus at this stage of development.

[16] C. C Congdon, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p 88.

Table 26. Effect of Fetal Thymus on Mortality from Secondary Disease

Host	Number of Donor Cells Injected Intravenously		Number of Mice	Number Dead on Day			Mortality (%)
	HBM on Day 2	Fetal Thymus on Day 0		30	60	90	
	($\times 10^6$)	($\times 10^6$)					
LAF_1/Cum ♂ ♂			37	36	37	37	100
Recipient	10 A·CA		46	12	38	42	91
950 r	10 A·CA	27–68 A·CA	16	6	12	13	81
$1C3F_1$/Cum ♂ ♂			60	59	59	59	98
Recipient	10 $B6D2F_1$/Cum		59	3	20	30	51
950 r	10 $B6D2F_1$/Cum	10–85 A·SW	13	2	7	7	54

AN AUTORADIOGRAPHIC STUDY OF THE GERMINAL CENTER IN THE SPLEEN WHITE PULP DURING EARLY INTERVALS OF THE IMMUNE RESPONSE

M. G. Hanna, Jr.

Introduction. – Histological evidence has pointed to the germinal centers of lymphatic tissue as functional structures in the immune response. As described by Congdon and Goodman,[17] one of the most dramatic histopathological changes observed in the lymphatic nodules of mouse spleen during the early intervals of a primary immune response is the germinal center dissociation. A view presented by Congdon[18] is that the dissociated germinal center cells are actively proliferating and differentiating into recognizable members of the plasma cell series.

At present there is considerable evidence that the precursor to the plasma cell is a primitive lymphatic tissue cell. Microscopic observations of early intervals of the primary and secondary immune response relate the increase of the latter cells in lymphatic tissue to germinal center dissociation. However, no attempt has been made to establish quantitatively that the primitive lymphatic tissue cells concentrated in germinal centers do migrate out to surrounding areas in normal or immunized animals.

The particular inquiries of this study were the quantitative aspects of germinal center dissociation and the migration of germinal center cells in normal and antigenically stimulated animals. The spleen was chosen for this study.

Materials and Methods. – Three groups of male LAF_1/Cum mice, 15 to 16 weeks of age, were used in this study. In group A, 20 mice were injected with 1 ml of the 10% sheep red blood cells (SRBC) and another 20 were given an equal volume of Tyrode's solution. One hour later, all animals received 50 μc of H^3-thymidine. Four SRBC-stimulated and four control animals were killed at 2, 4, 6, 8, and 10 hr after the antigen or Tyrode's injection.

The same experimental procedure was followed for groups B and C. In group B the isotope was injected at 9 hr after the antigen or Tyrode's, and the animals were killed at 10, 12, 14, and 16 hr. In group C the H^3-thymidine was injected at 17 hr after antigen or Tyrode's, and the animals were killed at 18, 20, 22, and 24 hr.

Results and Discussion. – The results of these autoradiographic studies indicate the following:

1. In SRBC-stimulated and control animals the cells of the densely populated side of the germinal centers are precursors to the cells of the adjoining, thinly populated region, and the latter cells migrate into the lymphocyte mass.
2. The doubling time (synthesis + G_2 + prophase + metaphase + anaphase) of dividing cells in the germinal centers (those in the densely populated region) is 5 to 7 hr, while the cells of the thinly populated region and those dispersed throughout the associated lymphocyte mass double in about 9 hr.
3. The germinal centers in control animals demonstrate a steady-state system, in which cells are entering and leaving each compartment at a constant rate. In SRBC-stimulated animals, evidence for disruption of this steady state is manifested by an increased number of dividing cells and an increase in the number of cells migrating into the lymphocyte mass. This response ultimately results in complete germinal center dissociation.

The observed dissociation of germinal centers in the first 24 hr of the primary immune response can now be postulated as a direct or an indirect disruption by the antigen of a steady-state compartment of primitive lymphatic tissue stem cells. These cells then disseminate throughout the spleen and undergo intense proliferation. On a morphologic and dynamic basis there is no reason to doubt that a correlation exists between the germinal center cells and the antibody-forming cells which are a constant feature of the histology of the immune response.

PHAGOCYTIZED PLASMA CELLS IN THE SPLEEN OF IMMUNIZED MICE

D. C. Swartzendruber

An intense plasmacytopoiesis occurs in lymphatic tissue following antigenic stimulation.

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[17] C. C Congdon and Joan Wright Goodman in *Proceedings of International Symposium on Tissue Transplantation* (ed. by A. P. Cristoffanini and G. Hoecker), pp 181–207, University of Chile, Santiago, 1962.

[18] C. C Congdon, *J. Natl. Cancer Inst.* **28**, 305 (1962).

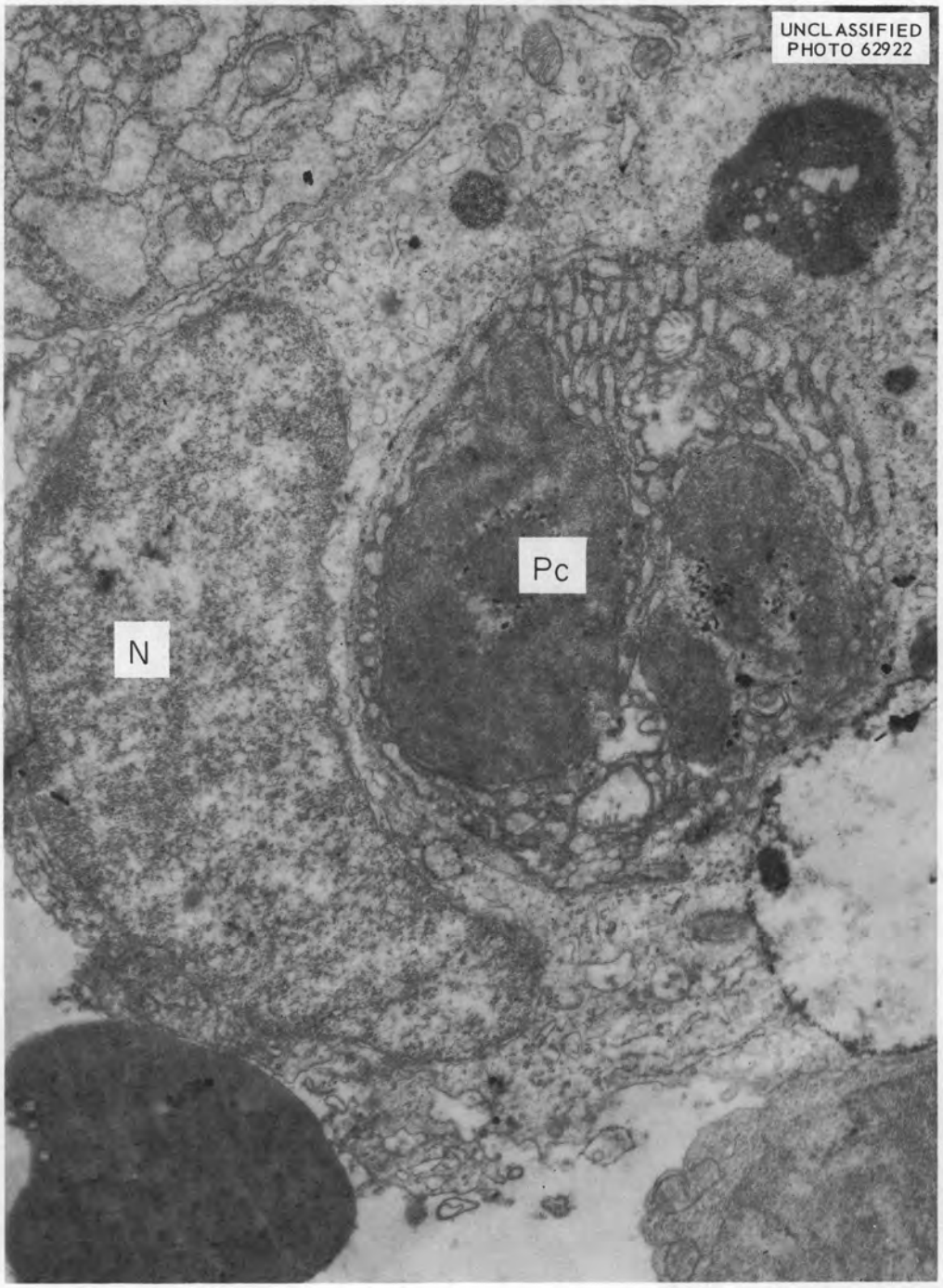

Fig. 23. Splenic Macrophage from Red Pulp of Spleen Four Days After Injection of Sheep Erythrocytes. Phagocytized plasma cell (*Pc*) and nucleus (*N*) of macrophage are labeled. 12,000X.

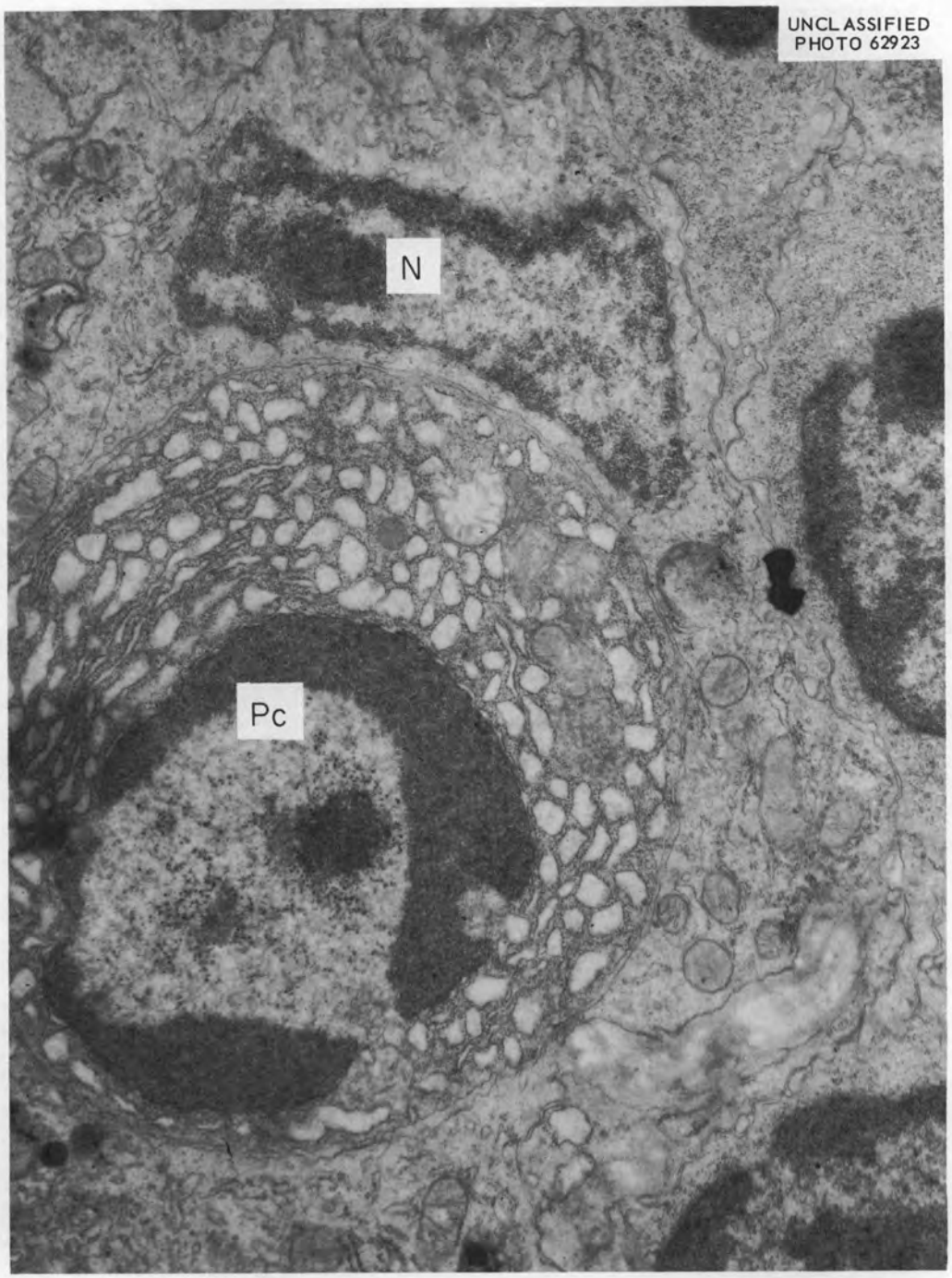

Fig. 24. Reticular Macrophage from White Pulp of Spleen Six Days After Injection of Sheep Erythrocytes. Nucleus (*N*) of macrophage and engulfed plasma cell (*Pc*) are labeled.

The origin, structure, and function of plasma cells evoked during an immune response have been widely studied. Relatively little information is available concerning the fate of plasma cells. Fagraeus[19] noted that the number of immature plasma cells observed in the spleen three to four days after stimulation exceeded the number of mature plasmacytes found seven to ten days later. Similar observations have been reported by other investigators. Dedifferentiation, transformation, migration, and death of plasma cells have been suggested to account for their disappearance. Electron microscopic observations reported here suggest that many immature plasma cells are phagocytized in the mouse spleen during the log phase of antibody production three to seven days after a single intravenous antigen injection.

Materials and Methods. – Five groups of ten adult $(101 \times C3H)F_1$ mice received a single intravenous injection of 1 ml of Tyrode's containing either 10^8 rat bone marrow cells or 2×10^9 sheep erythrocytes. Spleens were removed at daily intervals after injection and fixed for light and electron microscopy.

Results and Discussion. – Phagocytosis of plasma cells in the spleen was first observed on day 3. During the log phase of antibody production, days 3 to 7, phagocytized plasma cells were noted with increasing frequency. Macrophages in both red and white pulp (Figs. 23 and 24) contained plasma cells in varying stages of digestion. On day 10, mature plasma cells were observed in the spleen, with no evidence of recognizable phagocytized plasma cells. Electron microscopy does not reveal the significance nor extent of this mode of plasma cell removal from the spleen. Phagocytosis of plasma cells as described in this report may represent an essential process in the immune response or the removal of cells which have discharged their function. In either case, support is indicated for the view that plasmacytes are terminal cells. The extent of plasma cell migration from the spleen under conditions of these experiments is unknown.

[19] A. Fagraeus, *Acta Med. Scand., Suppl.* **204**, 1 (1948).

RADIOSENSITIVITY OF ERYTHROPOIETIC COMPARTMENT OF MOUSE BONE MARROW

L. H. Smith

Introduction. – Radiation sensitivity of a proliferative cell system can be defined best in terms of reproductive capacity. In lethally irradiated mice transplanted with isologous bone marrow, this capacity can be measured by spleen uptake of Fe^{59}.

Methods. – The protocol of the assay has been described.[20] Lethally x-irradiated mice were injected with bone marrow exposed *in vitro* to 0, 100, 200, 300, or 400 r. A linear response of uptake is obtained at each x-ray exposure, a greater number of cells being required as the exposure increases. The number of cells required to effect an arbitrary percentage uptake can be determined at each x-ray exposure. The percentage of surviving cells can then be calculated at each *in vitro* exposure by dividing the number of normal cells required to give the arbitrary percentage uptake by the number of irradiated cells needed to give an equivalent uptake.

[20] L. H. Smith and T. W. McKinley, Jr., this report.

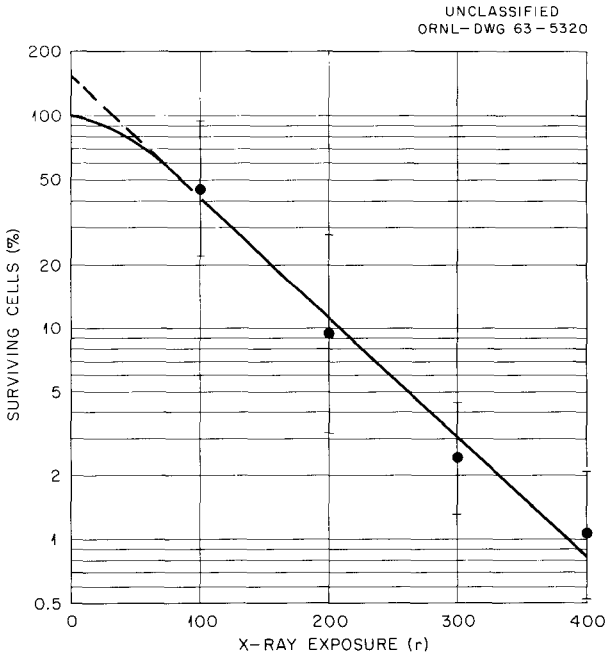

Fig. 25. Effect of *in Vitro* X Irradiation on Erythropioetic Compartment of Bone Marrow. Vertical bars indicate 95% confidence intervals.

Results and Discussion. – The percentage of surviving cells after *in vitro* x irradiation is shown (Fig. 25). From the linear portion of this curve, the amount of radiation required to reduce the number of surviving cells to 37% (D_{37}) is 76 r. The extrapolation number is 1.6. These values compare favorably with those obtained by Hodgson,[21] who used erythrocyte Fe^{59} as a measure of erythropoietic capacity rather than spleen uptake of this isotope.

SPLEEN UPTAKE OF Fe^{59} AS A MEASURE OF REPRODUCTIVE INTEGRITY OF TRANSPLANTED BONE MARROW CELLS

L. H. Smith T. W. McKinley, Jr.

Introduction. – Evaluation of Fe^{59} uptake by mouse spleen as a method for measuring reproductive capacity of bone marrow cells transplanted in lethally irradiated recipients has continued.[22] The major variables of the assay are herein described.

Results. – Comparison of intravenous, intraperitoneal, and subcutaneous routes were made. No route was superior insofar as consistency of uptake was concerned. The percentage uptake of Fe^{59} is independent of isotope dose in the range 0.05 to 0.8 μc per mouse. Uptake was not altered by different methods of killing (luxation, ether, and decapitation).

The sooner after marrow injection that the Fe^{59} is given, the greater the number of cells required to effect a linear response. Day 7 or 8 is satisfactory since a small number of cells (0.05 to 0.8 million) is sufficient to produce linearity over about the same cell dose range where 30-day survival proceeds from 0 to 100%.

The percentage of Fe^{59} incorporated is a function of the time after isotope injection. Incorporation increases during the first 6 hr, levels off for the next 6 hr, and then gradually falls. Using the plateau interval of 6 to 12 hr is both advantageous and convenient.

A 6-hr spleen uptake of Fe^{59} was studied as a function of x-ray exposure. In mice that did not receive marrow, uptake was normal on day 8 after irradiation up to 425 r, followed by a decrease with increasing exposure. Since slight residual erythropoiesis occurred at 700 r, a safe exposure (one at which there is no significant host contribution) is 800 r or more. Injection of marrow enhanced erythropoiesis at the 300 to 600-r level; no significant change occurred thereafter to 950 r.

Plasma clearance of Fe^{59} is a function of bone marrow cell dose. This function was operative at all times from 30 min to at least 16 hr. Plasma Fe^{59} reached a maximum about 1 hr after injection of the isotope, and by 48 hr, it was very low.

Conclusions. – As a result of the foregoing studies, the following procedure has been used: mice are exposed to 850 r of whole-body x rays; 24 hr later they are injected intravenously with isologous bone marrow; 8 days later they are given 0.1 μc of Fe^{59} intraperitoneally; and 6 hr thereafter they are killed by luxation, and the spleens are removed for radioactivity analysis.

DYE EXCLUSION BY BONE MARROW CELLS IN SHORT-TERM CULTURE

Karen T. Bredahl

Introduction. – The inability of cells to resist dye uptake is generally assumed to be an indication of cell injury, loss of normal cellular functions, and cell death. Dyes such as eosin Y, nigrosine, and trypan blue are used extensively for this purpose. We are interested in the use of these dyes as a means of estimating the integrity of mouse bone marrow following short-term cell culture and in comparing the dye exclusion technique with a method that measures reproductive capacity of bone marrow cells.

Materials and Methods. – Suspensions of bone marrow cells from mouse femurs were prepared in Tyrode's, and the uptake dynamics of several dyes were tested, including nigrosine and lissamine green V as well as eosin Y. Dye concentration and exposure time were investigated. Since eosin Y gave the most consistent results, further studies with cultured marrow cells were performed with this dye. Marrow cells were suspended in a variety of media and incubated under different conditions. The variables were media,

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[21] G. S. Hodgson, *Acta Physiol. Latinoam. Am.* 12, 365 (1962).

[22] L. H. Smith and T. W. McKinley, Jr., *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 72.

pH, temperature, container, and degree of agitation. The eosin Y uptake by cells was determined over a 6-hr culture period. The degree of cell clumping and the decrease in total cell count were also recorded.

Results and Conclusions. – Of the dyes tested on fresh bone marrow preparations, eosin Y uptake was most consistent and less dependent on dye concentration and time of exposure. The average zero time eosin Y uptake was (17.7 ±1.0)%. In a sealed hemocytometer uptake increased slowly during the first 20 min and then more rapidly, reaching 100% in about 2 hr. In a test tube suspension, uptake remained essentially constant for 1 hr. Regardless of dye concentration, nigrosine-positive cells were not so prominent as those containing eosin Y; therefore no further studies were made with this dye. Lissamine green V had the disadvantage of showing a rapid increase during the entire time of exposure to the dye in a sealed hemocytometer. On the other hand, negligible uptake occurred in a test tube suspension over a 3-hr period. Results of these exploratory experiments indicated that eosin Y was preferable to lissamine green V or nigrosine.

A correlation was found between both increased temperature and increased violence of agitation and the increased dye uptake; however, gentle agitation was somewhat better than none at all and was markedly preferable to vigorous agitation (Table 27). The incubation medium of 0.9% NaCl proved to be the poorest. Addition of glucose

Table 27. Environmental Effects on Eosin Y Uptake and Total Cell Count over a 6-hr Period for Mouse Bone Marrow Suspensions

Medium	Temperature (°C)	Type of Agitation	Increase of Eosin Y Uptake (%)	Decrease of Total Cell Count (%)	Number of Experiments
0.9% NaCl	24	*a*	51	7	2
Tyrode's solution	24	*b*	1	23	5
Tyrode's solution without $CaCl_2$	24	*b*	35	30	2
	24	c	29	30	4
TC 199 with 50% calf serum	24	*a*	9	22	3
0.9% NaCl	37	*a*	243	10	2
0.9% NaCl with 50% calf serum	37	*a*	79	22	2
Tyrode's solution	37	*b*	50	59	2
Tyrode's solution without $CaCl_2$	37	*b*	113	38	3
	37	None	68	19	2
	37	*a*	63	27	3
Tyrode's solution without $CaCl_2$ with 50% calf serum	37	*a*	65	22	6
TC 199 with 25% calf serum	37	*a*	76	13	5
	37	*d*	48	11	3
TC 199 with 50% calf serum	37	*a*	50	14	2
	37	*d*	0	14	2

[a]Gently swirled at 30-min intervals for 5 sec.

[b]Subjected to vortex agitation at 30-min intervals for 5 sec.

[c]Continuous rotation at 12 rpm.

[d]Continuous rotation at 1 rpm.

and other nutritive agents in Tyrode's solution resulted in less dye uptake over the 6-hr period, and TC 199 with 10 to 25% calf serum was found to be the most adequate medium. The pH did not appear to be extremely critical during incubation; however, a pH of 7.2 was more desirable than extremely acid or alkaline suspensions. Of the containers tested the 25-ml Erlenmeyer flask with ground-glass stoppers seemed to be the best for incubation suspensions of 6 to 8 ml. Regardless of environmental conditions, cell clumping increased and total cell counts decreased over the 6-hr period, but neither correlated with the increase in dye uptake.

A comparison was made of the percentage decrease of nonstaining cells with eosin Y and the decrease in activity obtained with the spleen iron assay.[23] In two experiments it was found that the average decrease of cell viability over a 6-hr period with the dye uptake method was 8%, while the spleen iron assay decrease was 34%. From this we concluded that these two methods do not reflect the same mode of damage to the marrow cells.

ANIMAL TESTING PROGRAM

M. L. Simmons[24]

Introduction. – A health check is made on incoming animals to be used for research in the Biology Division. Extensive testing is done for *Pseudomonas* and *Salmonella* infections in mice, rats, and hamsters. In addition, spot checking for internal and external parasites is carried out, and rabbits and some other animals are examined as they are received. The data obtained from these studies are sent to the animal supplier and the investigator. Object testing of equipment, water bottles, personnel, and working areas for *Pseudomonas* and *Salmonella* contamination is routinely performed. Animals with unexpected illnesses in the Experimental Animal Facility are examined at the request of the investigator.

Methods. – Composite fecal samples are taken from each cage of mice, rats, and hamsters soon after arrival in the receiving building and are examined by bacteriological techniques for *Pseudomonas* and *Salmonella*. One mouse from each of 10 cages in a major strain is sampled for parasites each week.

Wet, sterile swabs taken of equipment, water bottles, personnel, and working areas are incubated in brain-heart-infusion broth for 48 hr and then streaked on glycerol and brilliant-green agar for detection of *Pseudomonas* and *Salmonella*. *Salmonella* typing is also done.

Results and Discussion. – The results on *Salmonella* and *Pseudomonas* testing of fecal samples over a five-week period are shown in Fig. 26. Animals in all cages giving positive tests were killed unless special handling was undertaken.

Parasite checks on LAF_1/Cum and C3H/Anf/Cum mice were negative.

Object testing results are not yet complete; but, as in the past, *Pseudomonas* was usually present in wet areas around the cage and bottle washers. Clean water bottles were negative for *Pseudomonas*. The high *Pseudomonas* level in the first week of July was caused partly by a single contaminated rat shipment. One major source of inbred mice consistently showed high *Pseudomonas* contamination, and shipments were discontinued from this supplier.

The problem resulting from *Pseudomonas* in mice used for radiation studies in Oak Ridge and its control by proper water bottle handling procedures has been reported by Woodward.[25]

[23] L. H. Smith and T. W. McKinley, Jr., this report.

[24] Experimental Animal Facility.

[25] J. M. Woodward, *Laboratory Animal Care* 13, 20 (1963).

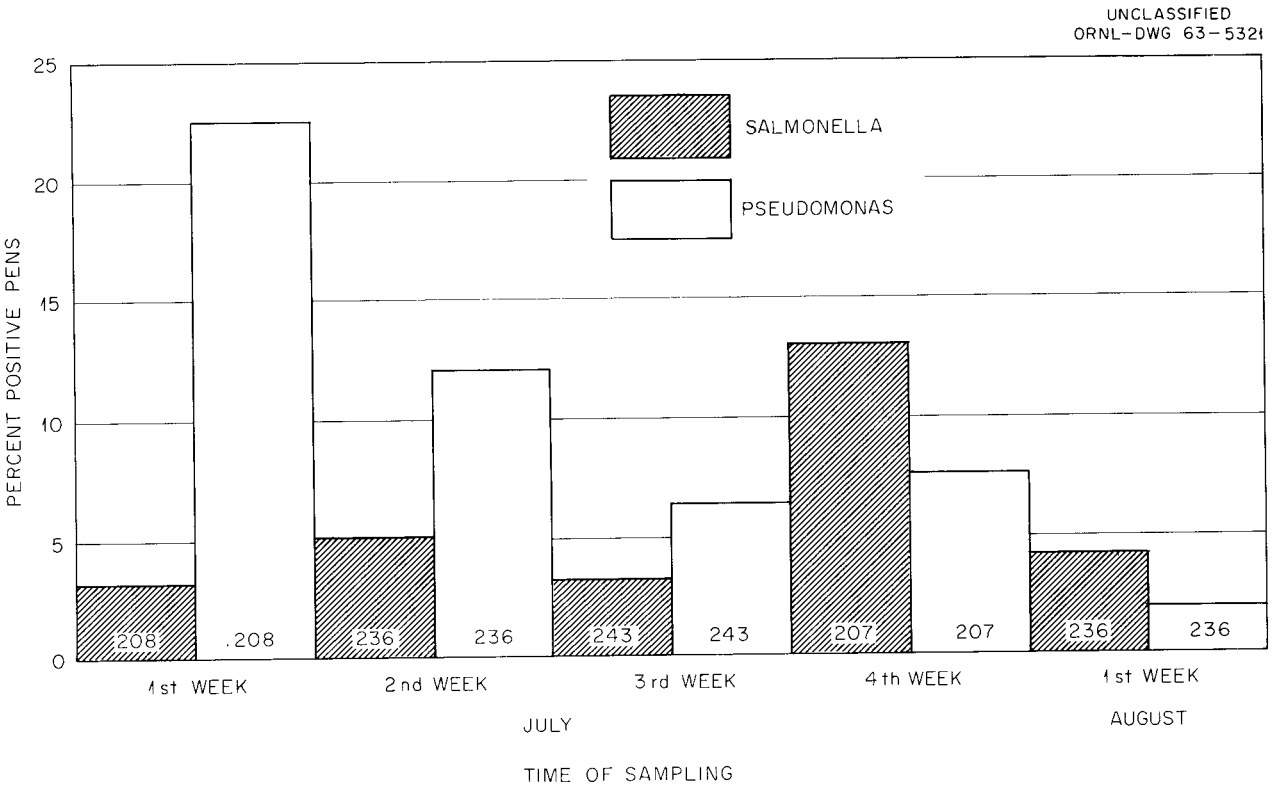

Fig. 26. Percentage of Positive Pens Obtained from Composite Fecal Samples in a Five-Week Period. The numbers at the bottom of the graph show the total number of cages sampled.

Tissue Culture

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IRRADIATION-INDUCED POLYPLOIDY IN LEUKOCYTE CULTURES

A. G. Bell

In cultures of human leukocytes polyploidy occurs in two forms: tetra- or octaploid cells and cells with endoreduplicated chromosomes. These phenomena can be induced by irradiation of the cultures. There are wide variations in polyploid incidences (3 to 20%) appearing in different experimental preparations that are presumably subjected to the same conditions of culture and irradiation. Variables were introduced into culture conditions in an effort to determine the cause or causes. Many of the factors tested showed no apparent effect. These included x-ray dose rate, chilling (to 5°C) at the time of irradiation, maintaining the temperature of the culture at 40°C, and the use of autologous versus inactivated AB serum in the medium. The concentration of phytohemagglutinin, however, significantly affected the polyploid incidence, although not enough to explain the wide variations.

Two-wavelength microspectrophotometric studies were undertaken to show that endoreduplication is definitely a form of polyploidy with doubled cell deoxyribose nucleic acid (DNA) content, rather than just caused by split chromatids in cells with the 2c DNA values. The DNA content in nine diploid cells was measured as 431 ± 18 arbitrary units and in five endoreduplicated cells was 829 ± 90 units.

Earlier work[1] supported the concept of Levan and Hauschka[2] that endoreduplicated cells yielded tetraploid daughter cells at mitosis. The present study, however, shows that tetraploid cells are also produced directly. The early appearance of tetraploid cells following irradiation, in addition to the fact that duplicate chromosome aberrations appear in them, indicate that both the early tetraploid cells and the endoreduplicated cells are a result of the same phenomenon. Both types of cell, therefore, appear to result from endoreduplication: in those cells destined to become typical tetraploid cells, the diplochromosome halves separate, whereas they do not in the cells appearing endoreduplicated at metaphase.

[1]A. G. Bell and D. G. Baker, *Can. J. Genet. Cytol.* **4**, 340 (1962).

[2]A. Levan and T. S. Hauschka, *J. Natl. Cancer Inst.* **14**, 1 (1963).

Two cycles of replication of the interphase chromosome, rather than one, occur to produce these polyploid cells. Different observations provide suggestive evidence that these cycles are contemporaneous rather than consecutive. Polyploid cells appear as early as 24 hr after irradiation and as early as 64 hr following preparation of the culture. Autoradiographic studies of the earlier stages of culture (12 to 18 hr) indicate that there is no irradiation-induced DNA synthesis which could provide for consecutive cycles of DNA replication. Further, in a few endoreduplicated cells from cultures provided with tritium-labeled thymidine 5 hr after irradiation, we have observed the presence of both two- and four-chromatid labeling in different diplochromosomes of the same cell. This is taken to support the view of contemporaneous cycles of DNA replication.

KINETICS OF ABERRATION LOSS IN THE LEUKOCYTES OF IRRADIATED HUMANS

M. A Bender P. Carolyn Gooch

Introduction. – We have previously reported studies of aberration levels in the peripheral leukocytes of accidentally irradiated normal humans.[3–6] More recent data make it possible to draw certain conclusions about the kinetics of aberration loss from the peripheral circulation during the first five postirradiation years.

Materials and Method. – Additional material, obtained at approximately five years postirradiation, has been scored from the eight men involved in the "Y-12 accident." New samples have been scored from the three men involved in another criticality accident.[5] These were obtained at 6 weeks, 3½ months, 6 months, and 1 year postirradiation. Additional samples obtained at eight months postirradiation from four men accidentally exposed to gamma irradiation[6] have also been scored. In all cases the peripheral leukocyte chromosome techniques used to prepare the previous samples were employed. As previously, approximately 100 cells were scored visually from each sample, and approximately 10 karyotypes were prepared from superficially normal cells.

Results and Discussion. – The gross results from the present samples, together with those reported earlier, are presented graphically in Figs. 27–29. It is apparent that the frequencies of chromosomal aberrations fluctuate from sample to sample. Some of this fluctuation can be ascribed to normal processes of cell replacement, but some must be due to temporal fluctuations in the contribution of various sources of peripheral leukocytes (e.g., the temporary "success" of particular clones of cells). Individual "C" in in Fig. 27 and "A" in Fig. 28 are notable examples of such fluctuation. In spite of the fluctuation, however, certain changes of a regular nature appear in the data. For the first few weeks after irradiation the aberration levels remain fairly constant. During this period, all cells with

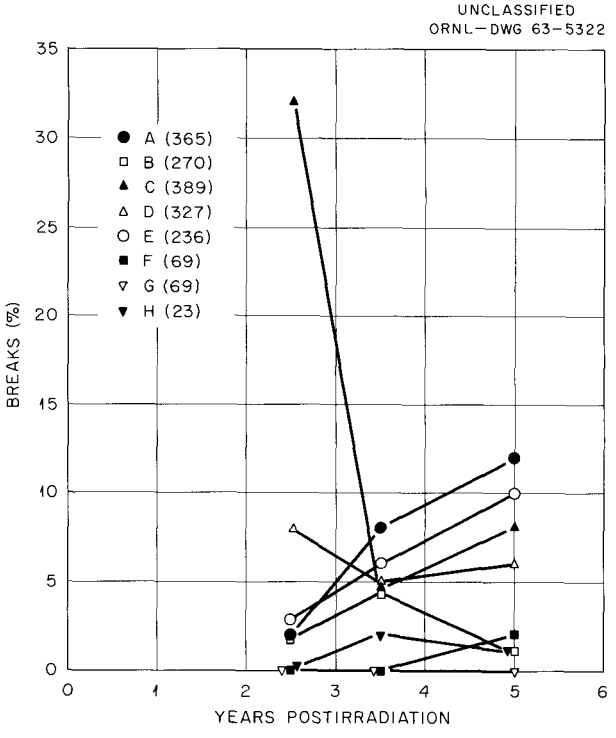

Fig. 27. Percentage of Chromosome Breaks in the Eight Men Involved in the "Y-12 Criticality Accident." Estimated dose (in rads) given in parentheses.

[3]M. A Bender and P. C. Gooch, *Radiation Res.* **16**, 44 (1962).

[4]M. A Bender and P. C. Gooch, *Radiation Res.* **18**, 389 (1963).

[5]M. A Bender and P. C. Gooch, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, pp 195–97.

[6]M. A Bender and P. C. Gooch, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 156–57.

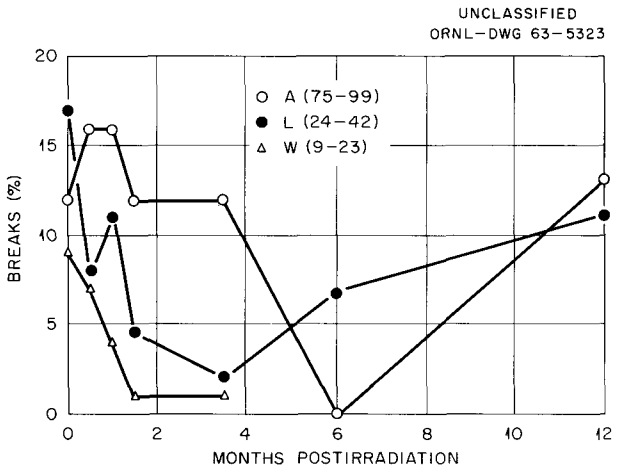

Fig. 28. Percentage of Chromosome Breaks in Three Men Accidentally Exposed to Whole-Body Irradiation from Mixed Gamma Rays and Fission Spectrum Neutrons. Estimated dose (in rads) given in parentheses.

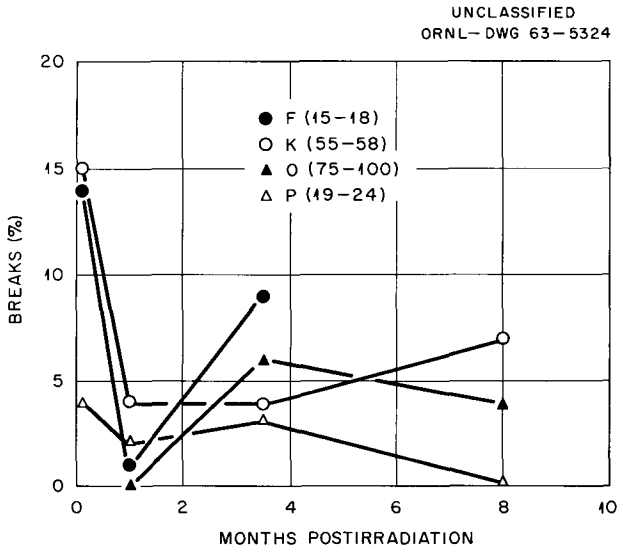

Fig. 29. Percentage of Chromosome Breaks in Four Men Accidentally Exposed to Whole-Body Gamma Irradiation. Estimated dose (in rads) given in parentheses.

aberrations also have the expected acentric fragments. Evidently, if cells are replaced at all, they are replaced by differentiation, without division. At about one month, aberration levels begin to fall quite sharply, and at the same time cells appear which lack the expected acentric fragments. It appears that the replacement of circulating leukocytes is by cell division during this period. These suggestions are further supported by the results of karyotype analyses of superficially normal cells. During the first few postirradiation weeks, the only aberrations picked up are symmetrical translocations and pericentric inversions. When the aberration level begins to fall, however, deleted chromosomes, which are not recognized in gross scoring because the fragments are missing, begin to show up on karyotype analysis. For the first few months after the fall begins, all the aberrant cells appear to have undergone at least one cell division postirradiation. In the 6- to 12-month samples, however, cells in their first division again appear. The gross scoring data suggest an increase from the low aberration levels at 1 to 2 months, which may be explained by this reappearance of first-division cells.

Although it is evident that much more frequent sampling of future material will be required to elucidate the exact kinetics, the present results are consistent with the following:

1. the existence of a period of 3 to 4 weeks postirradiation, during which the aberration level stays constant with no replacement by cell division;
2. the replacement of circulating cells by cell division during the 3- to 5-week period postirradiation;
3. a period, including 6 to 12 weeks, when few if any cells which have not divided postirradiation are present in the circulation;
4. a period, extending at least from 6 months to 5 years postirradiation, during which some cells which have not divided since irradiation again appear in the circulation.

ABERRATION RATES INDUCED BY 14-Mev NEUTRON IRRADIATION OF HUMAN BLOOD *IN VITRO*

P. Carolyn Gooch M. A Bender
M. L. Randolph

Introduction. – Uncertainty about the RBE for chromosomal aberration production and the kinetics of two-hit aberration production by neutrons of various energies has hampered previous efforts to make accurate dose estimates from peripheral leukocyte chromosome aberration frequencies in cases of whole-body exposure of humans during

criticality accidents.[5] Studies of the effects of RBE = 1 x rays and of 14-Mev, 2.5-Mev, and fission spectrum neutrons have been undertaken; the experiments with 14-Mev neutrons and with RBE = 1 x rays have been completed.

Method. – Freshly drawn whole human blood was irradiated in nylon chambers. The irradiated leukocytes were separated, cultured, fixed, mounted, and stained by the techniques used in our previous x-ray studies.[7] The experiments were repeated with blood from three different donors. The neutrons were generated by the D,T reaction, using a Cockcroft-Walton generator. The x-ray exposures, made to confirm that our earlier experiments did indeed represent RBE = 1, were made under conditions similar to those used for the neutron exposures. The x-ray parameters were 250 kvp; hvl, 2 mm Cu; dose rate, 10 rads/min.

Results and Discussion. – Table 28 shows the results of the x-ray experiments. Table 29 gives the results for the 14-Mev neutrons. Because the results for the three individuals were not significantly different, they are pooled in both tables. Surprisingly, the ring and dicentric frequencies from not only the x-ray experiments, but also the

[7]M. A Bender and P. C. Gooch, *Proc. Natl. Acad. Sci. U.S.* **48**, 522 (1962).

Table 28. X-Ray-Induced Aberrations in Human Blood Irradiated *in Vitro*

Dose (rads)	Cells Scored	Cells with $2n \neq 46$	Chromatid Aberrations	Chromosome-Type Aberrations					
				Deletions	Rings	Dicentrics	Exchanges	Breaks[a] (%)	Rings and Dicentrics (%)
Control	411	13	3	1	0	0	0	0.2	0
25	300	19	5	11	0	1	2	4.3	0.3
50	300	22	2	17	0	5	9	9.0	1.7
100	500	46	9	32	0	29	25	18.0	5.8
200	400	55	10	75	3	95	25	67.7	24.5

[a]Deletions plus twice the dicentrics; inversions, etc., are excluded because of the subjective nature of their determination.

Table 29. Neutron-Induced Aberrations in Human Blood Irradiated *in Vitro*

Dose (rads)	Cells Scored	Cells with $2n \neq 46$	Chromatid Aberrations	Chromosome-Type Aberrations					
				Deletions	Rings	Dicentrics	Exchanges	Breaks[a] (%)	Rings and Dicentrics (%)
Control	300	30	7	0	0	0	0	0	0
50	287	22	5	32	3	12	11	21.6	5.2
100	300	19	3	48	5	23	13	36.0	9.3
150	290	29	8	89	9	52	13	72.8	21.0
200	200	37	3	97	4	60	11	114.5	33.0

[a]Deletions plus twice the dicentrics; inversions, etc., are excluded because of the subjective nature of their determination.

14-Mev-neutron experiments, showed excellent fits to dose-squared kinetics. Coefficients of aberration production were calculated from the expressions $Y_1 = a_1 + bD$ and $Y_2 = a_2 + cD^2$, where Y is the yield, a is the coefficient of spontaneous aberrations, b is the coefficient of deletion production, c is the coefficient of ring and dicentric production, and D is the dose. The coefficients are presented in Table 30, together with those calculated from the previous x-ray experiments. It is apparent that the two sets of x-ray data are in very good agreement, in spite of the rather different conditions under which the two sets of experiments were conducted. The coefficients for the 14-Mev neutrons, on the other hand, are significantly different from the x-ray coefficients. The RBE for deletion production is 2.6; that for ring and dicentric production is only 1.4. This large a difference in RBE is somewhat unexpected. If it is only a sampling error, the average RBE for 14-Mev neutrons for chromosomal aberration production in the human leukocyte system is 2.0.

Table 30. Coefficients of Aberration Production for X Rays and 14-Mev Neutrons

Irradiation	Deletions per Cell per Unit Dose	Rings and Dicentrics per Cell per Unit Dose^2
	$\times 10^{-2}$	$\times 10^{-5}$
X rays (60 r/min)	0.11 ±0.012	0.52 ±0.07
X rays (10 r/min)	0.09 ±0.003	0.60 ±0.05
14-Mev neutrons	0.23 ±0.022	0.81 ±0.06

Radiation Immunology

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RELATIVE POOL SIZE OF POTENTIALLY COMPETENT CELLS AS A FUNCTION OF TIME AFTER ANTIGEN INJECTION

Takashi Makinodan W. J. Peterson

Introduction. – Although it is known that the relative pool size (RPS) of potentially competent (PC) antibody-forming cells will increase and then, in general, decrease with time after an administration of a priming dose of antigen, quantitative kinetic information essential to our understanding of the suicidal differentiation process of somatic cells is lacking. In view of the changing RPS of PC cells of the spleen of normal mice with respect to age,[1] we felt that as a preliminary study mice should be primed with an antigen at an age when the RPS of PC cells of their spleen is beginning to increase logarithmically. Therefore, for this study, 1-month-old (C57 × C3H)F_1 mice were primed intraperitoneally with 2×10^8 rat RBC antigen, and the RPS of PC cells in a fixed number of spleen cells was assessed in *in vivo* cultures at monthly intervals for two years. A fixed number of spleen cells from *non-primed* mice of comparable age groups were used as "background" controls.

Results. – As shown in Fig. 30, the RPS of PC cells of the spleen of normal nonprimed mice increase about 20-fold between 1 and 4 months of age, decreased about 4-fold between 5 and about 15 months of age, and remained stationary between 15 and 30 months of age. In contrast, a 20-fold increase in the RPS of PC cells of the spleen was observed in less than 1 week after exposure of 1-month-old mice to a priming antigen and a 100-fold increase in 30 days. After reaching its maximal level five months after antigenic stimulation, the RPS of PC cells in the spleen decreased about 2-fold within a month before decreasing with a $T_{1/2}$ comparable to that of the control group.

[1]T. Makinodan and W. J. Peterson, *Proc. Natl. Acad. Sci. U.S.* **48**, 234 (1962).

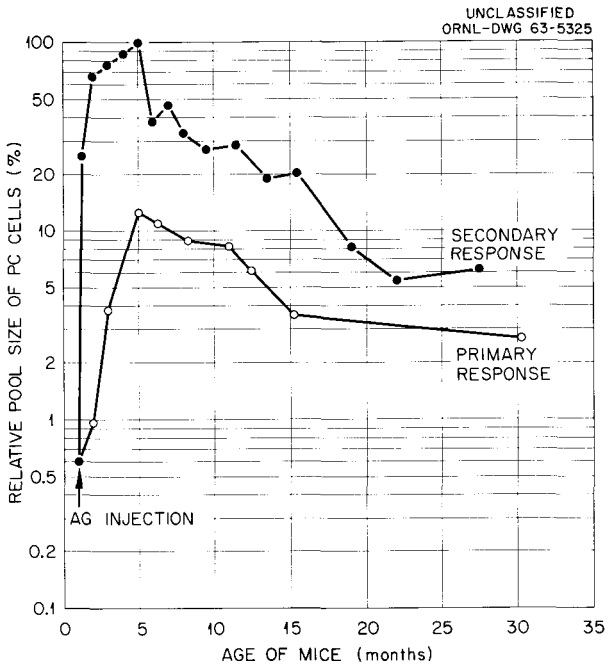

Fig. 30. Change in the RPS of PC Cells as a Function of Time After Antigen Stimulation.

Discussion. – These results show that, although there is an accelerated increase in the RPS of PC cells in the spleen during the first 30 days after antigenic stimulation, the rate of decrease shortly after reaching its maximal level is comparable to that observed among aging normal mice.

DECAY OF POTENTIALLY COMPETENT ANTIBODY-PRODUCING CELLS FOLLOWING PRIMARY ANTIGEN STIMULATION

J. F. Albright T. W. Evans

Introduction. – It was reported previously[2] that there is competition of non-crossreacting antigens during the latent phase of primary antibody response. In view of the rapid proliferation and differentiation of cells involved in antibody production following antigen triggering, we believe that the competitive effect is due to temporary depletion of the limited number of potentially competent (PC_1) cells which can be stimulated by antigen at any given time. The present study is concerned with the rate, extent, and duration of PC cell depletion.

Methods and Results. – Twelve-week-old (C3H × 101)F_1 mice were injected intravenously with 2×10^9 sheep erythrocytes (Srbc). At intervals thereafter, they were again injected with 2×10^8 rat erythrocytes (Rrbc). Blood serum samples were collected at daily intervals for a period of ten days following injection of Rrbc. They were titrated for antibody to Rrbc, and the antibody titer estimated at the end of the logarithmic phase of the response was selected as the most reliable single point for comparison. Figure 31 is a logarithmic plot of antibody titer as a function of the time (t) between administration of Ag_1 (Srbc) and Ag_2 (Rrbc). It is seen that the duration

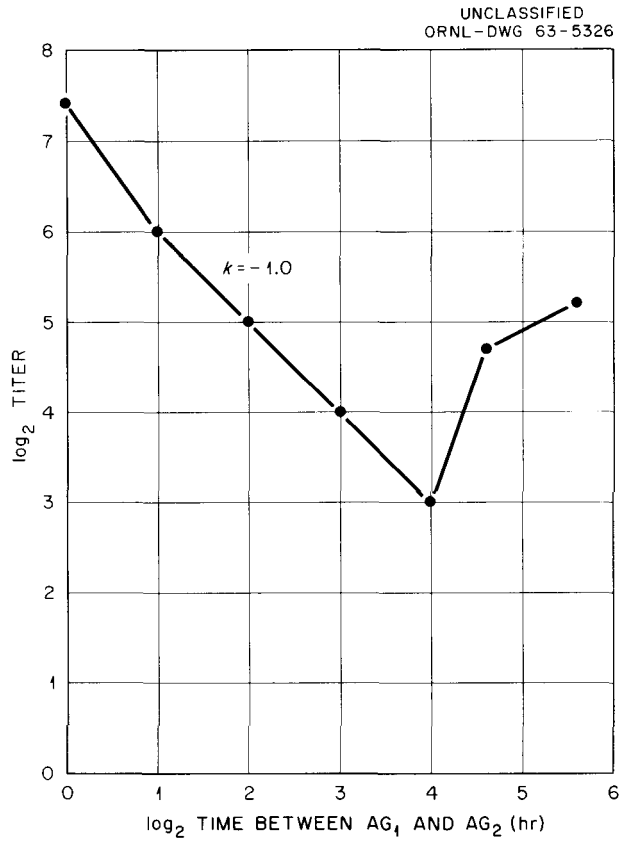

Fig. 31. Antibody Response to Rrbc, Indicative of Relative Number of PC_1 Cells, as a Function of Time After Priming with Srbc.

[2]T. Makinodan and W. J. Peterson, *Biol. Div. Semi-ann. Progr. Rept. Feb. 15, 1963, ORNL-3427, p 84.*

of the decay phase is 16 hr, followed by the beginning of the recovery phase. The decay constant, k, is estimated to be -1.0. Owing to the relation between titer and cell number,[3] the decay of PC_1 cells can be characterized by the expression $N = N_0 t^k$.

Discussion. – The factors influencing the decay in numbers of PC_1 cells following primary antigen stimulus are, as yet, poorly understood. They probably include (1) the time of persistence and concentration of the initial antigen, (2) the rate at which antigen-stimulated cells are removed from the PC_1 (multipotential) compartment, and (3) the rate at which the PC_1 compartment is replenished. It seems clear, however, that the initial events in antibody response involve multipotential cells.

FATE OF PC_2 CELLS DURING PROLONGED *IN VIVO* CULTURE

E. H. Perkins

Introduction. – In recent experiments[4] the relative pool size (RPS)[5] of potentially competent antibody-forming cells from primed donors (PC_2 cells) neither increased nor decreased significantly when cultured in lethally irradiated isologous recipients for five days. A slight decrease in RPS was noted during the second week of culture. This decrease was not significant. In view of the fact that the rate of decrease in the RPS of PC_2 cells may be gradual, estimates were made at weekly intervals for seven weeks using the procedure described previously.

Results. – It can be seen in Fig. 32 that there was a small, but progressive, decrease in pool size during the first two weeks of culture of 4×10^7 PC_2 cells. A further decrease was noted during the subsequent two weeks of culture and became significantly reduced before stabilizing at a level of ~25 to 30% of the initial pool size. This plateau cannot be attributed to the returning immunological activity of the host, since this response accounts for less than 5% of the total pool size. Preliminary investigations, using 2.5×10^6 PC_2 cells, suggest that the rate of decline in pool size may be related to the initial inoculum size. Studies are in progress to determine whether or not further decrease in the RPS of PC_2 cells can take place when spleen cells are cultured *in vivo* for periods extending beyond seven weeks.

Discussion. – These results are consistent with the postulate that PC_2 cells develop as a result of antigenic stimuli, have a fixed differentiation path, and are insensitive to hematopoietic or other unrelated antigenic stimuli. The reduction in the RPS of PC_2 cells during the first four weeks of culture suggests that, if these cells are dividing symmetrically with a constant generation time, then the rate of output of these cells from this compartment exceeds the rate of input into this compartment.

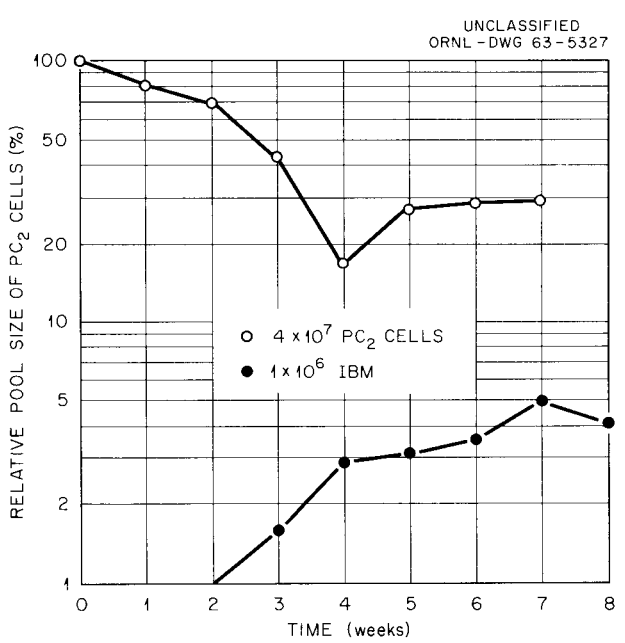

Fig. 32. Reduction of the RPS of PC_2 Cells During *in Vivo* Culture.

[3] T. Makinodan and J. F. Albright, *Third International Symposium on Immunopathology*, in press.

[4] E. H. Perkins and T. Makinodan, *J. Immunol.*, in press.

[5] $RPS = \{1/[2^{(\log_2 \text{titer}_{to} - \log \text{titer}_{tx})}]\} \times 100.$

QUANTIFICATION OF THE RELATION BETWEEN ANTIGEN DOSAGE AND SECONDARY ANTIBODY RESPONSE BY MOUSE SPLEEN CELLS CULTURED IN DIFFUSION CHAMBERS

Immo Hoppe

Introduction. – Previous experiments[6] have shown a proportionality between the antibody titers of the stationary phase of response and the concentration of antigen. For further studies on the cellular events accompanying immune reactions of varying magnitude, it seemed desirable to do a full-scale experiment concerned with antigen-antibody relations in diffusion chambers in which, owing to an effect of reaction volume, it is possible to obtain a spectrum of response ranging from extreme antigen insufficiency to extreme antigen excess.

Method. – Spleen cell donors were female $C31F_1$/Cum mice, 19 weeks of age, which had been primed at the age of 13 weeks with one single intraperitoneal injection of 1 ml of a 1% suspension of sheep red blood cells (Srbc) (equivalent to 2.6×10^8 cells). Twelve million spleen cells were placed in diffusion chambers together with varying numbers of Srbc ranging from 12×10^3 to 12×10^8, such that the ratio (spleen cells/Srbc) in the various groups decreased in tenfold steps from 1000:1 to 1:100. The chambers were implanted into $C31F_1$/Cum mice of both sexes (10 to 18 weeks old), which had been irradiated before the operation with 600 r. The experiment was performed with one pool of spleen cells and one sample of Srbc. Serum and chamber fluid titers of each of the six groups were determined from samples collected on days 2, 4, 6, 8, 10, and 12 of culture.

Results and Discussion. – The antibody production of the different groups was evaluated by comparing the $\log_2$ titers of the stationary phases. Optimal conditions were obtained when the ratio of spleen cells to Srbc was 1:0.1, the average titers of the stationary phase (days 8, 10, 12) being 12.9 in the chamber fluid and 10.9 in the serum. Whereas the corresponding titers of the neighboring groups with ratios of 1:10, 1:1, and 1:0.1 were appreciably less, in the case of extreme antigen excess (ratio 1:100) and extreme antigen insufficiency (ratio 1:0.001) the differences were especially pronounced. In antigen excess, titers reached only 6.6 in the chamber fluid and 5.4 in the serum; the induction period was prolonged to four to five days. In the antigen-limiting situation, the induction period was approximately normal, while the titers of the stationary phase were identical with those of the antigen excess system. These data are shown in Fig. 33, where the titers of the stationary phase in terms of relative activity are plotted against the ratio of spleen cells to Srbc. Here it is seen that the difference of ~6 in $\log_2$ titer between optimal conditions and extreme conditions

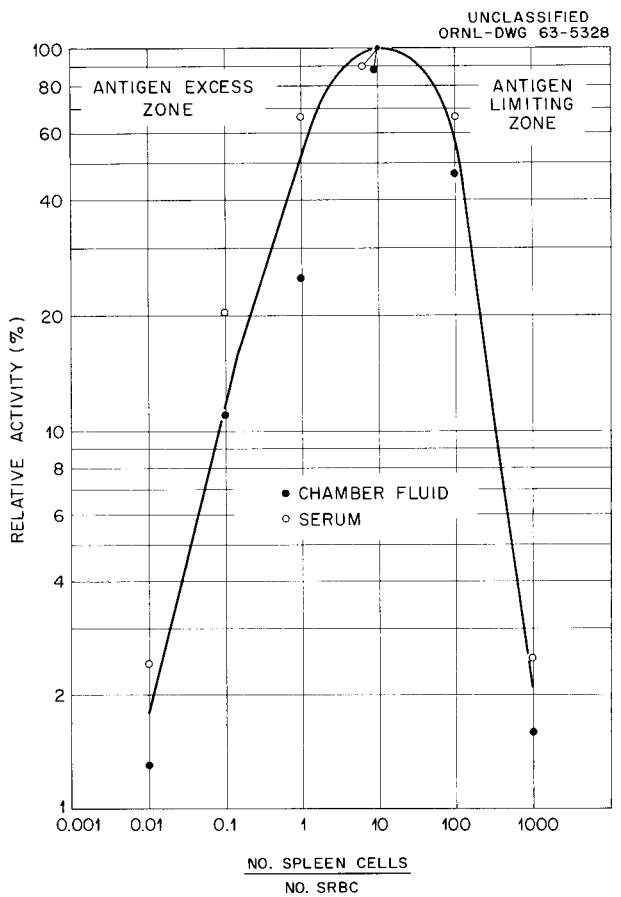

Fig. 33. Relation Between Antibody Production of the Stationary Phase of a Secondary Response (in Terms of Relative Activity) and the Ratio of Spleen Cells to Sheep Red Blood Cells. The number of spleen cells was kept constant at 12×10^6 per chamber. The number of sheep red blood cells varied between 1.2×10^9 and 1.2×10^4 per chamber.

[6] T. Makinodan and J. F. Albright, *Third International Symposium on Immunopathology*, in press.

means that only ~1.5% of the amount of antibody which can be produced by the number of cells cultured is detected where antigen is either in severe shortage or in great excess.

From the relation $Ab = (\text{constant})(Ag)^{k_2}$, where the number of cells is fixed, k_2 for the region of antigen excess is estimated as –0.6. Extrapolation of the data indicates that a condition of complete failure of antibody production may be obtained at a spleen cell to Srbc ratio of approximately 10^4 (antigen shortage) and approximately 10^{-5} (antigen excess).

ESTIMATION OF GENERATION TIME OF BLAST CELLS DURING LATE LATENT PHASE OF ANTIBODY RESPONSE

Toshihiko Sado Martha R. Leonard

Introduction. – Since previous observations have suggested a short generation time of blast cells during the latent phase of antibody production, we have focused our attention on the cell cycle of blast cells during this period. When primed rabbit spleen cells, along with the secondary dose of antigen, are cultured in *in vivo* diffusion chambers in the mouse, the number of blast cells reaches a state of dynamic equilibrium three to four days after the culture has started. Therefore, we have estimated the duration of various stages of the blast cell cycle during this period. This was done by determining the frequency of labeled mitotic cells as a function of time after a single pulse injection of H^3-thymidine.

Results and Discussion. – The result is presented in Fig. 34. In this experiment, prophase was not included in the mitotic cells because of the inability to reliably distinguish it from the resting stage. Therefore, in this experiment the post-DNA synthetic phase (G_2) is the interval between the end of DNA synthesis and metaphase.

1. The time between addition of H^3-thymidine and the appearance of the first labeled mitotic figure is the time between the end of DNA synthesis and the onset of mitosis. This is about 45 min.

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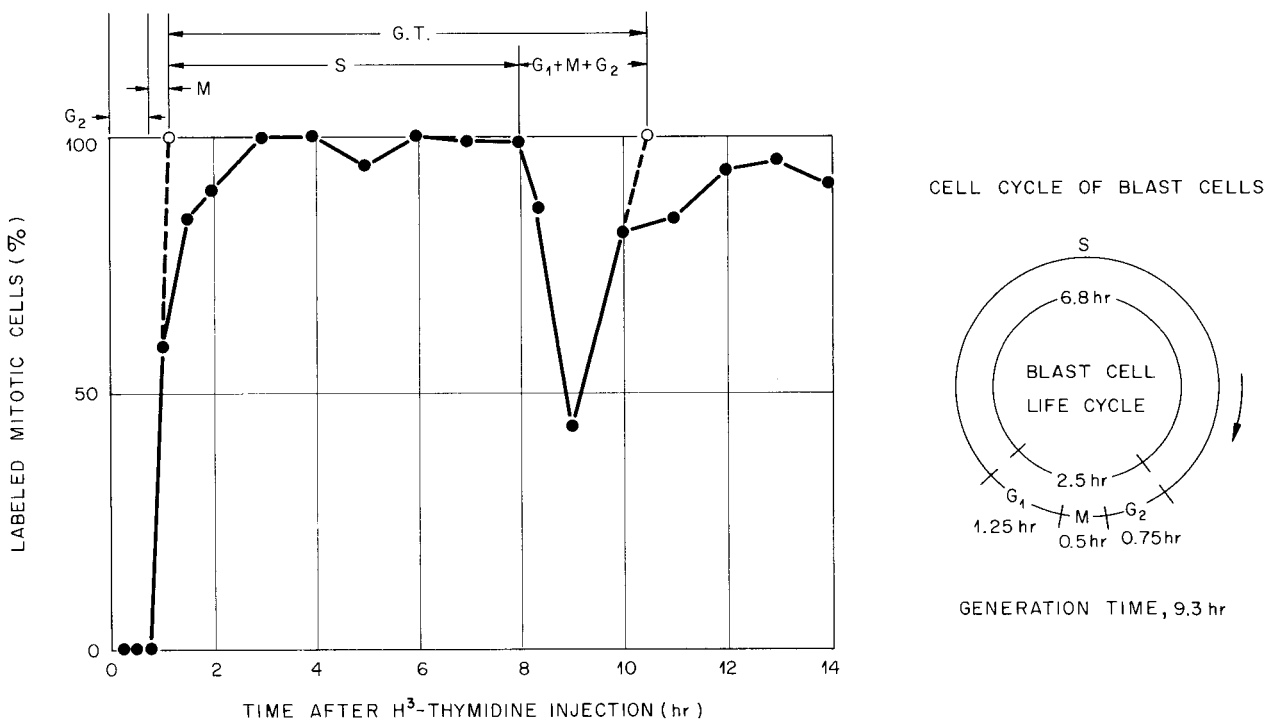

Fig. 34. Percentage of Label in Blast Cell Mitotic Figures as a Function of Time After a Single Pulse Injection of H^3-Thymidine.

2. The percentage of labeled mitoses rises from 0 to 60% in 15 min, suggesting that mitotic time (M) is approximately 30 min. The duration of time for the percentage of labeled mitoses to increase to approximately 100% is a reflection of the variability of time of G_2 and M phases. Considering this, we have extrapolated the first rising curve to the 100% level. This point is 1.2 hr after H^3-thymidine administration.

3. The time during which all mitotic figures are labeled is an estimate of the minimum DNA synthesis period (S). If we accept the above extrapolation, the curve from 1.2 to 8 hr is approximately 100%. The curve begins to fall somewhat later than 8 hr; thus some cells in pre-DNA synthetic period (G_1) must have entered mitosis by 8 to 8.3 hr; thus the minimum S time must be 8 – 1.2, or 6.8 hr.

4. The interval between the time when the percentage of labeled mitoses in the first generation reaches 100% and the time when the mitoses in the second generation are 100% labeled represents the total generation time of this particular cell type. By extrapolating the second rising curve, we assume that the time when the labeled mitoses reach 100% in the second cycle is ~10.5 hr. Thus, the generation time of these cells must be 10.5 – 1.2, or 9.3 hr.

5. The time of the G_1 phase can be obtained by subtracting duration of G_2, M, and S phases from the total generation time. This is estimated as ~1.25 hr.

CHANGES IN TOTAL SPLEEN CELL NUMBER ASSOCIATED WITH EMBRYONIC SPLENOMEGALY

Frank Seto

Introduction. – Although indirect evidence[7] indicates that splenic enlargement which follows the grafting of immunologically competent tissues is the result of cell proliferation, actual estimates of cell numbers of embryonic spleens undergoing splenomegaly would provide direct quantitative information. Twelve-day-old embryonated eggs were injected intravenously with adult chicken spleen cells (1 to 4 million); at time intervals thereafter the eggs were killed, the embryonic spleen was weighed, and cell suspensions were prepared and counted in a hemacytometer. Normal 8- to 18-day-old embryonated eggs were injected intravenously with sterile saline solution, and cell counts were made in a similar manner.

Results and Discussion. – The mean spleen cell counts per milligram of wet tissue weight of control embryos of 12 to 18 days of incubation were approximately 1.8×10^6 cells, but that for the 8- to 10-day-old embryos was about 1×10^6. In the experimental group, on the other hand, the cell count decreased progressively from 2×10^6 (in 12- to 14-day-old embryos) to a low value of 0.5×10^6 in 20-day-old recipients. The total cell count was computed by multiplying the cell count per milligram by the total spleen weight. The results are summarized in Fig. 35, where the time course changes in spleen weight and total cell

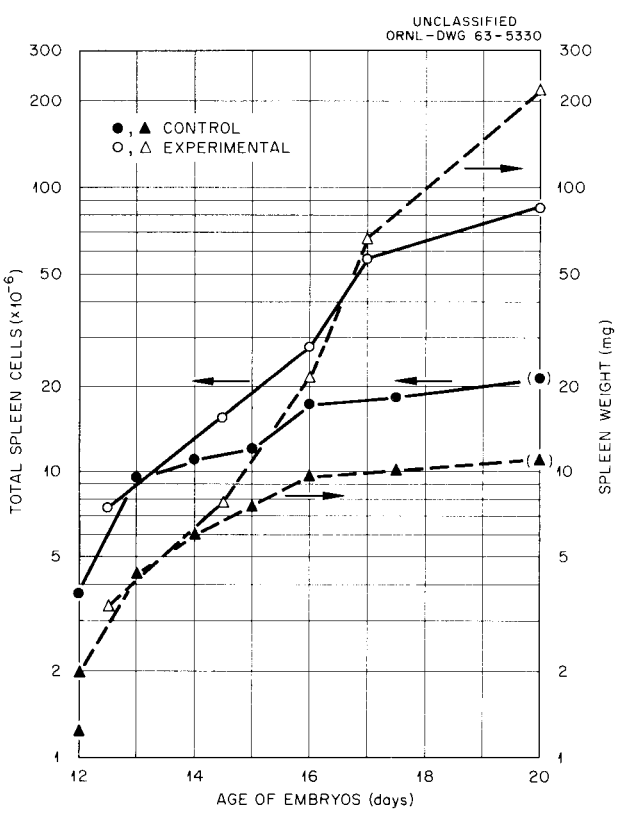

Fig. 35. Spleen Weight and Total Cell Count in the Embryonic Spleens. Total cell number: circles. Spleen weight: triangles.

[7] J. D. Ebert, *Proc. Natl. Acad. Sci. U.S.* 40, 337 (1954); J. B. Solomon, *J. Embryol. Exptl. Morphol.* 9, 355 (1961).

counts for both the control and experimental groups are represented. Although the number of cells per milligram decreased in the experimental group, the total number of splenic cells increased significantly, indicating that the splenic enlargement is due in part to increase in cell number. The apparent decrease in cell density suggests that there may be a shift in the relative proportion of larger cells. Preliminary differential studies of smears of cell suspension tend to support this opinion.

EFFECTS OF X IRRADIATION ON THE SPLENOMEGALY CAPACITY OF THE CHICK EMBRYO

Frank Seto

Introduction. – It is known that x irradiation reduces the immunological competence of graft tissue and the immune response capacity of recipient animals. As an extension of the quantitative study of splenomegaly in the chick embryo,[8] the effect of x irradiation on splenomegaly was investigated. Donor spleen cells and recipient embryos were exposed to varying amounts of x rays to determine the relative contribution of the two components in splenomegaly.

Methods. – Three series of irradiation experiments were performed in which (1) the host embryos, (2) the donor spleen cells, and (3) both host embryos and donor spleen cells were exposed to various amounts of x rays. Known numbers of spleen cells were injected intravenously, and the embryonic splenic enlargement was measured.

Results and Discussion. – The six-day spleen weights of irradiated embryos in both the controls and those injected with homologous cells were considerably less than their unirradiated controls. A comparable decrease occurred when donor spleen cells were irradiated. The results of these experiments are summarized in Fig. 36, in which the mean spleen weights of the unirradiated controls have been arbitrarily assigned a value of 100%. The decrease in spleen weights of the irradiated recipient embryos and of embryos injected with irradiated cells are given as relative values. It is clear from the similarity of the curves that irradiation of donor cells or recipient embryos affects splenic enlargement to the same extent. When irradiated hosts were injected with irradiated cells, the decrease was greater than when either donor cells or hosts alone were irradiated. The exposure to 600 r of both donor spleen cells and host embryos virtually eliminated the splenomegaly response. The data indicate that both host tissue and donor cells are contributing to the overall splenomegaly, and the similarity in the radiation exposure-response curves would suggest that the relative contribution is about the same.

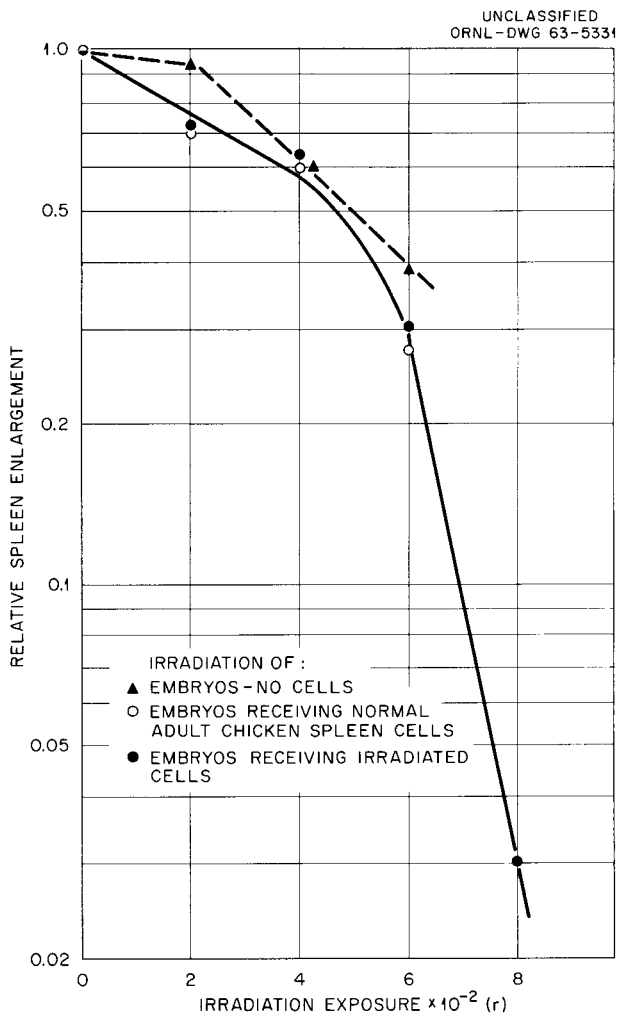

Fig. 36. Effect of Irradiation Exposure on Spleen Weight and Splenomegaly.

[8]F. Seto, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 86–87.

Pathology and Physiology

Section Chief – A. C. Upton

Pathological Effects of Neutrons and High-Energy Radiation in Mammals

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J. W. Conklin
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V. K. Jenkins
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EARLY HEMATOLOGIC CHANGES IN MICE EXPOSED TO 14-Mev WHOLE-BODY NEUTRON IRRADIATION

M. L. Davis E. B. Darden, Jr.
G. E. Cosgrove

A long-term study of mice exposed to 14-Mev neutrons from a Cockcroft-Walton accelerator is in progress. Some of the initial hematologic results of this study are summarized here.

Method and Results. – Young adult RF/Up female mice (10 to 11 weeks old) were exposed to 50, 100, 200, and 400 rads of 14-Mev neutrons produced by the D,T reaction in a Cockcroft-Walton accelerator. Control mice were sham-irradiated. Hematologic studies consisting of

total leukocyte counts, hematocrit determinations, and differential leukocyte counts were perfomed on five to ten mice from each treatment group, beginning 24 hr after exposure and continuing at irregular intervals thereafter. On the day after irradiation a marked reduction of total leukocyte and lymphocyte levels was seen, which was roughly proportional to dose (Figs. 37, 38). These levels remained low for 10 to 14 days, gradually rising thereafter. At 45 days after irradiation, however, the total leukocyte and lymphocyte levels had not returned to normal. Neutrophils were markedly depressed only in mice exposed to 100 rads or more, and neutrophil recovery occurred earlier, at lower dose levels, reaching approximately normal by day 14. The hematocrit did not depart from normal except in the group receiving 400 rads; in this group there was a gradual depression to approximately 90% of normal by the 10th day and a return to normal by the 26th day.

Discussion. – Although the neutron doses used were largely sublethal, there was a marked depression of circulating lymphocytes at all dose levels and of granulocytes at dose levels above 50 rads. The rapidity of recovery was dose-dependent and sufficient to prevent a significant fall in hematocrit. In general, the acute hematologic effects of 14-Mev neutrons appear similar to those of x rays in a comparable dose range.

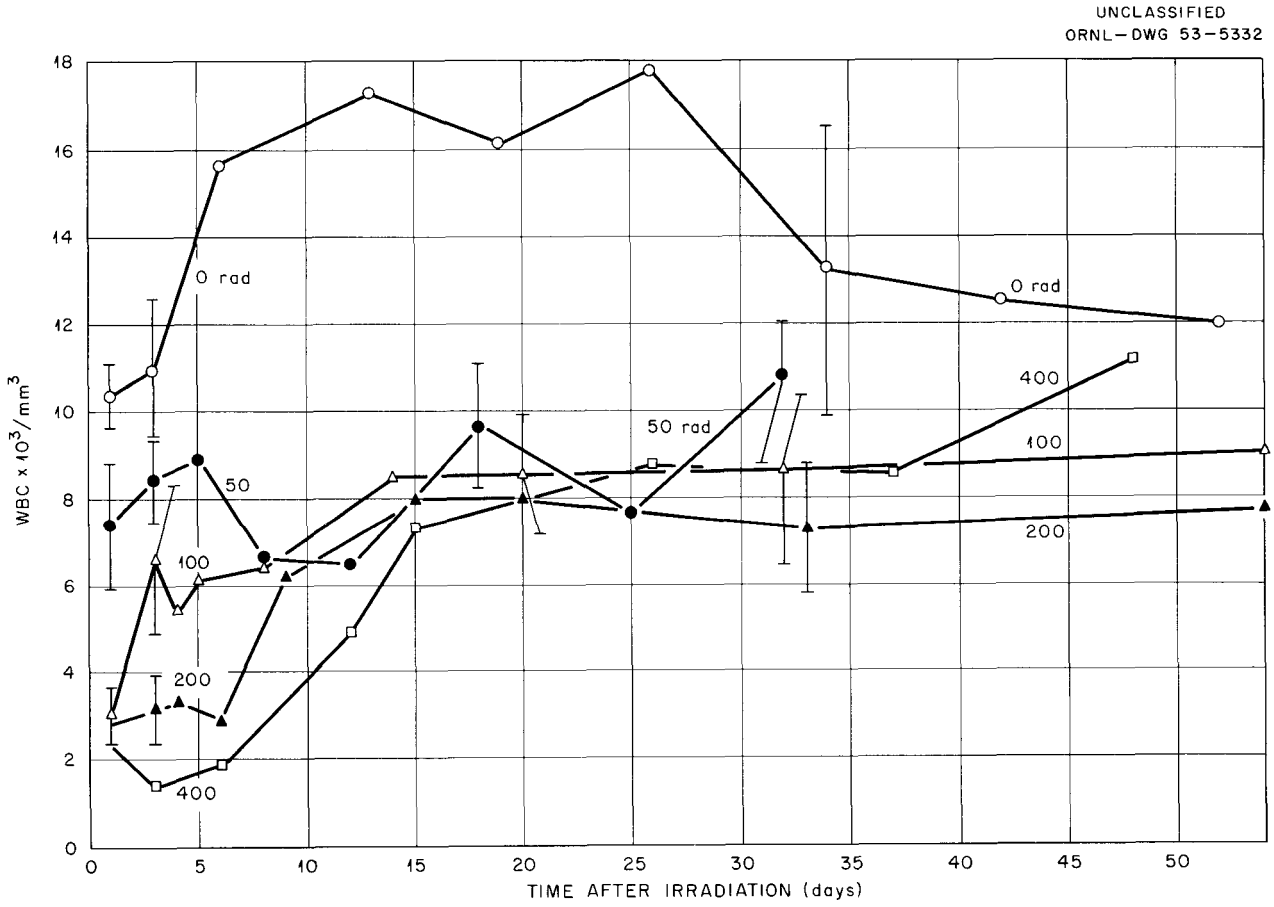

Fig. 37. Absolute Numbers of Total Leukocytes in Blood Following Irradiation with 14-Mev Neutrons. Vertical bars indicate 95% confidence range.

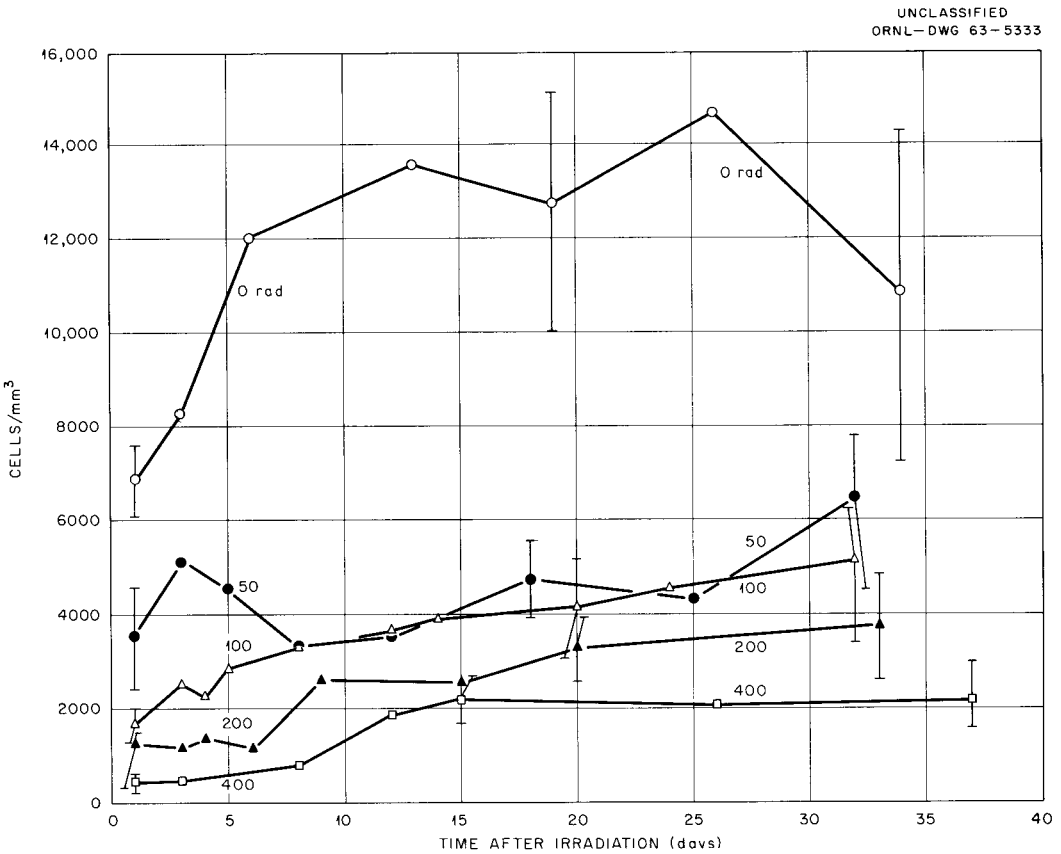

Fig. 38. Absolute Numbers of Total Lymphocytes Following Irradiation with 14-Mev Neutrons. Vertical bars indicate 95% confidence range.

INDUCTION OF CATARACTS IN MICE BY 4M2O

K. W. Christenberry J. W. Conklin
G. E. Cosgrove

Introduction. – The "radiomimetic" chemicals nitrogen mustard (HN2) and triethylene melamine (TEM) induce cataracts in female RF mice similar to those caused by ionizing radiation.[1] Studies[2] on 4-(*p*-dimethylaminostyryl) quinoline (4M2O) suggested the possibility that the "radiomimetic" effects of this drug might also include induction of lens opacities.

Methods and Results. – The 4M2O·HCl form of the compound, prepared at pH 1.5 as a 20% solution in distilled water, was administered intravenously into the tails of ten-week-old female RF/Up mice in doses of 0.075, 0.095, and 0.10 mg per kg of body weight. Control mice were injected intravenously with 0.2 ml of HCl at pH 1.5. The mice were housed in groups of ten per cage and allowed free access to food and water. An average of ten mice at each dose level was observed periodically for cataract formation; a complete description of the examination and scoring procedures has been reported.[3] The first indication of abnormality in the lens was detected in the 0.10 mg/kg dose group (Fig. 39), 87 days after treatment. This abnormality was a thickening of the anterior capsule and a diffuse granular appearance in the lens cortex, with vacuole formation at the anterior pole. The posterior suture line was clear. At dose levels lower than 0.10 mg/kg, the earliest changes involved the posterior pole of the lens. These were first detected 120 days after treatment in the 0.075 mg/kg dose group. Lens opacities sharply increased in severity after

[1] J. W. Conklin *et al.*, *Radiation Res.* **19**, 156 (1963).

[2] M. Asano *et al.*, *AMA Arch. Pathol.* **75**, 250 (1963).

[3] A. C. Upton *et al.*, *Cancer Res.* **20**, 1 (1960).

their onset in all treated groups. By the time senile opacities were first observed in the HCl-injected control mice, the 4M2O lesions were indistinguishable from radiation-induced cataracts of the same overall severity (Fig. 39). The mild opacities in the HCl-treated controls were similar in time of onset and rate of progression to those observed earlier in aging, untreated mice of the same strain.

Discussion. – This study indicates that 4M2O is strongly cataractogenic in mice. The relative effectiveness of this agent is indicated in the overall similarity of its effects to those induced by 500 to 600 rads of x irradiation (85 to 95 rads/min) or 280 rads of ~1.0-Mev fast neutrons (100 rads/min). Although 4M2O-induced cataracts were similar in severity to those induced by irradiation, this compound is significantly more cataractogenic than the "radiomimetic" agent Myleran, which from studies on rats[4] and mice[1] appears, in turn, more cataractogenic than HN2 or TEM at midlethal dose levels (Fig. 39).

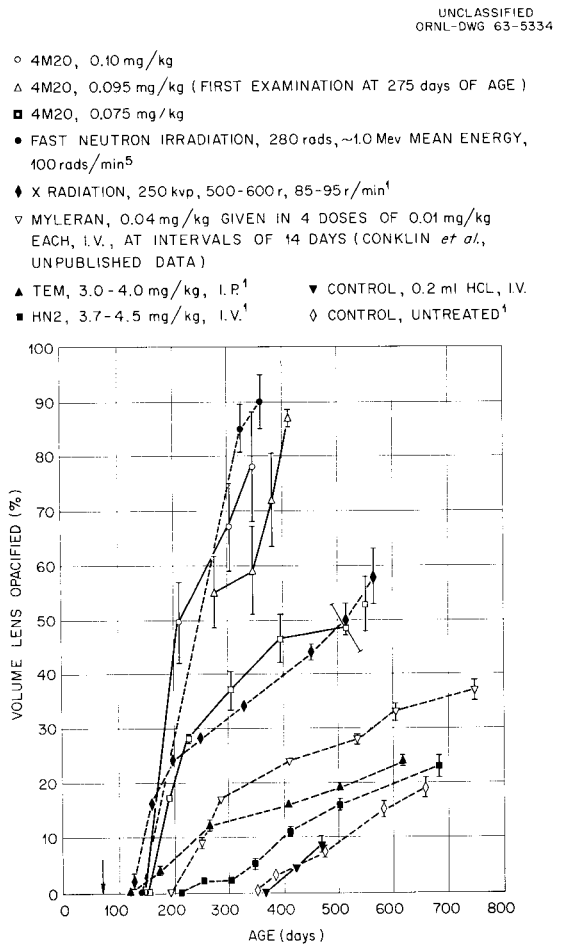

Fig. 39. Lens Opacities Induced by 4M2O and Other Agents in Relation to Time After Treatment. Arrow denotes age at treatment. Vertical bar indicates standard error of the mean at each point.

EFFECT OF TYPE AND RATE OF IRRADIATION ON INDUCTION OF DELAYED SOMATIC EFFECTS IN MICE

K. W. Christenberry J. W. Conklin
W. D. Gude

Introduction. – In a previous report the effectiveness of gamma rays for delayed somatic effects in mice was seen to vary inversely in relation to the dose rate.[5] The present study was undertaken to investigate the induction of delayed somatic effects by fractionated, as compared with daily, irradiation.

Results and Discussion. – Female RF/Up mice ten weeks old at the start of treatment were exposed to 995 to 1200 r of x or gamma rays at various dose rates (Table 31). The effects on survival (Fig. 40), most-frequent neoplasms (Table 31), and lens opacification (Fig. 41) varied inconsistently with the conditions of exposure. Although interpretation of the results is complicated by differences in the duration of the exposure period and in the dose and quality of the radiations, no one mode of treatment was uniformly more effective than the others. Fractionated, high-intensity x radiation yielded maximal results in the induction of myeloid leukemia and cataracts. In the induction of thymic lymphomas, however, daily gamma irradiation was more effective. Effects on survival were complex, reflecting an interplay of varying causes of mortality, yet to be fully analyzed. No consistent differences were noted in the incidence of ovarian tumors among the various groups, probably because all conditions of treatment exceeded the "saturation" level for induction of these neoplasms. The results with pulmonary adenomas are attributable to random variation, irradiation having previously been noted not to induce lung tumors in mice of this strain.

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[4] C. Solomon, A. E. Light, and E. J. DeBeer, *AMA Arch. Pathol.* 54, 850 (1955).

[5] J. W. Conklin and W. D. Gude, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1961*, ORNL-3095, pp 85–88, and unpublished data.

Table 31. Influence of Dose Rate and Fractionation of Irradiation on Longevity and Incidence of Major Neoplasms in RF/Up Mice

Type of Radiation	Dose Rate (rads/min)	Frequency of Treatment	Dose (rads)		Number of Mice Exposed	Mean Survival Time[a] (days)	Thymic Lymphomas		Myeloid Leukemias		Pulmonary Adenomas		Ovarian Tumor	
			Total	Mean			Incidence (%)	Mean Age[a] (days)	Incidence (%)	Mean Age[a] (days)	Incidence (%)	Mean Age[a] (days)	Incidence (%)	Mean Age[a] (days)
X rays	100	100 rads at 30-day intervals	1200	1066	112	430	25	362	23	444	21	475	53	469
X rays	80	300 rads at 14-day intervals	1200	1200	109	353	33	313	27	331	13	477	39	405
Co^{60}	0.016	Daily[b]	1012	1012	118	419	39	303	7	572	18	540	35	469
Co^{60}	0.011	Daily[b]	1020	1012	116	384	52	317	5	485	10	529	17	523
Co^{60}	0.0036	Daily[b]	1020	995	128	452	34	341	9	479	19	597	36	563
Control					1081	592	10	544	3	590	15	659	9	640

[a]Mean age at death.
[b]Exposed 23 hr/day.

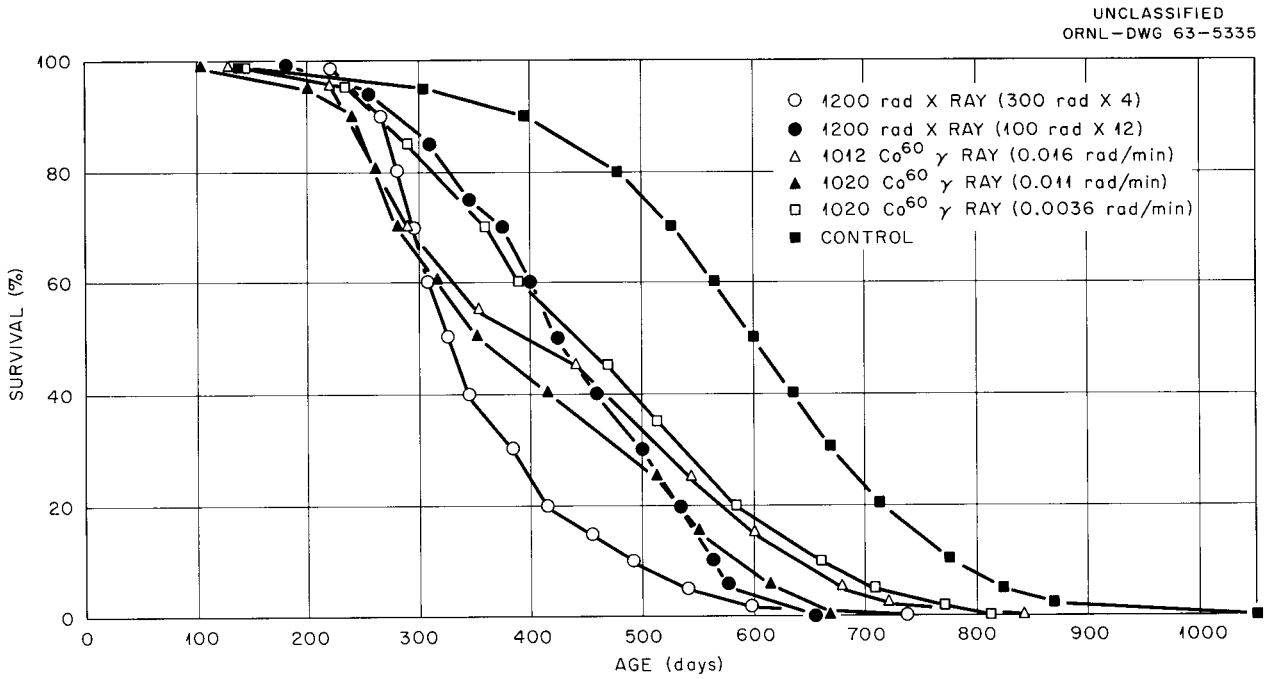

Fig. 40. Survival in Relation to Time After Irradiation and Conditions of Exposure.

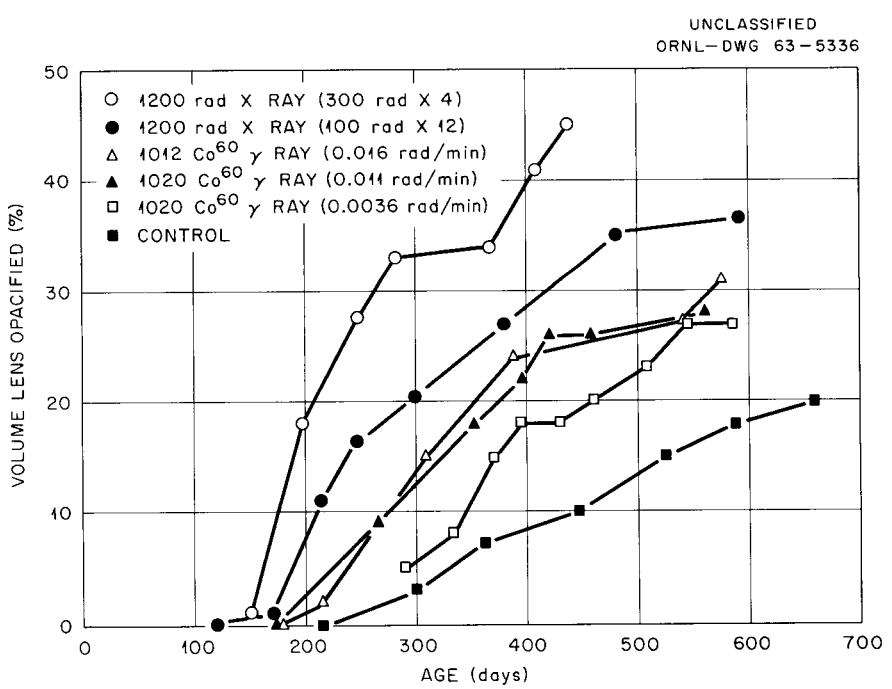

Fig. 41. Severity of Lens Opacities in Relation to Time After Irradiation and Conditions of Exposure.

PLATELET SIZE IN RELATION TO PLATELET AGE

T. T. Odell, Jr. T. P. McDonald

Introduction. – In an earlier study of the *in vivo* aging of rat blood platelets, the relative sizes of young, old, and normal populations of platelets were compared by measuring (with a cathetometer) the volume of packed platelets in microhematocrit tubes; it was found that platelet size decreased with age.[6] The purpose of this study was to investigate the frequency distributions of platelet sizes in populations of various ages using a Coulter counter with a particle-size-distribution plotter.

Methods. – Platelets were separated from the blood of male Sprague-Dawley rats using siliconized glassware and differential centrifugation in the cold. They were then resuspended in saline to give a concentration of 9747 to 37,030 platelets per 50 μl. The populations of young platelets were obtained from rats that were recovering from thrombopenia induced with antiplatelet serum.[6] Platelets were collected and sized two, three, and four days after antiserum injection. Populations of old platelets were prepared by incubating normal platelets in rats made platelet deficient as a result of the bone marrow aplasia produced by 900 r of whole-body x rays. Platelets were injected six days after irradiation and re-collected two days later. At this time, platelet counts of the transfused rats averaged 178,000 platelets/mm^3, and counts of rats similarly irradiated, but not transfused, averaged 31,700 platelets/mm^3. Platelet sizes were measured with a Coulter counter (model B) with a particle-size-distribution plotter. A

[6]T. C. Detwiler, T. T. Odell, Jr., and T. P. McDonald, *Am. J. Physiol.* **203**, 107 (1962).

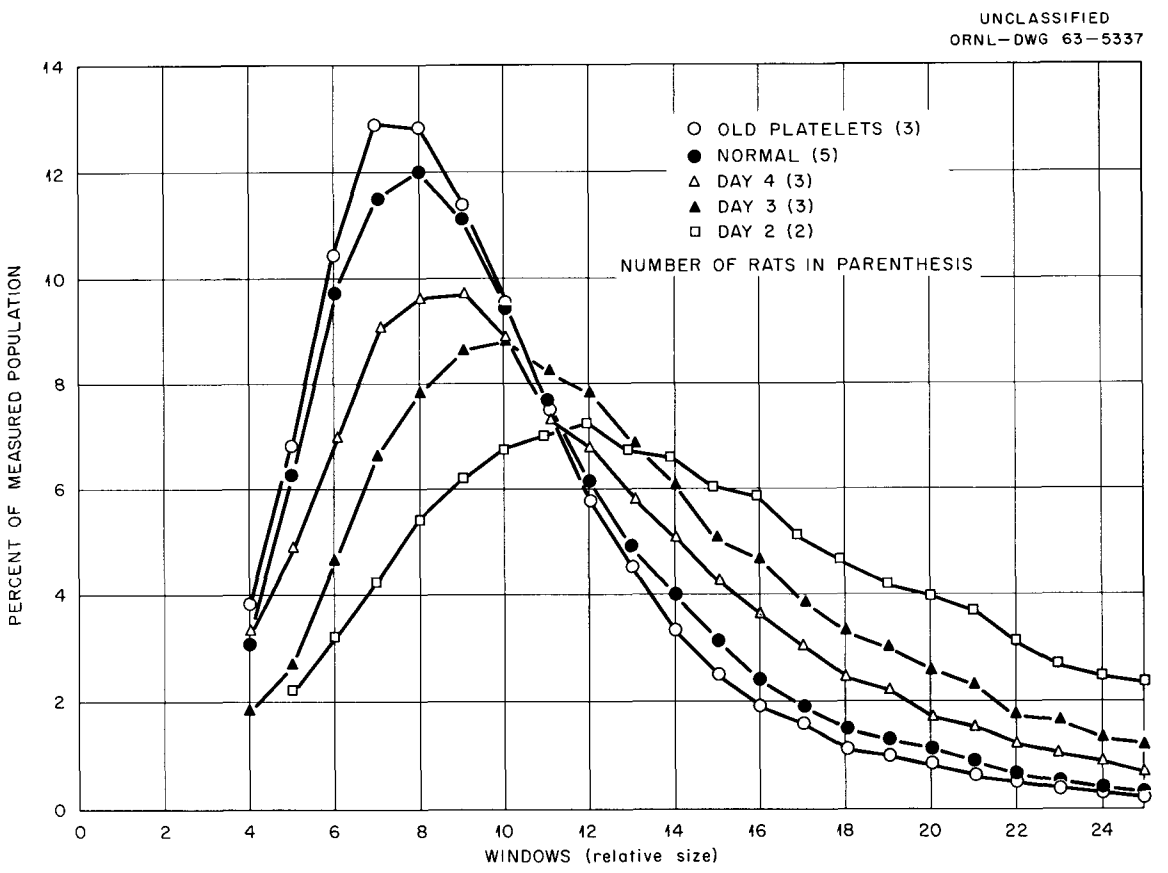

Fig. 42. Size Distribution of Platelets of Varying Age.

50-μ aperture was used, and the amplification and aperture current were each set at one-half. The particle size plotter automatically counts the number of pulses in a standard length of time (4 sec) in 25 successive four-unit pulse-height windows. The relative number of platelets per window is plotted directly in graph form, and a frequency distribution curve can be obtained by joining the peaks plotted at each window. After the platelets were sized, the plotter was disengaged, and the number of particles per 50 μl was determined. For numerical counts, the lower threshold was set at 5 and the upper threshold at its maximum, 100.

Results and Conclusions. – Peripheral platelet counts were reduced essentially to zero by platelet-specific antiserum, began to rise sharply one to two days later, and reached normal levels by day 4. The size distribution curve of platelets collected during recovery from the thrombocytopenia was shifted to the right at two days (indicating an increase in size) and then approached that of a normal population in the following days, as the number of platelets in the peripheral circulation approached normal values (Fig. 42). The size distribution curve of old platelets was shifted slightly to the left of the distribution curve of normal populations, indicating a somewhat smaller average size.

It is concluded that young platelets obtained in the manner described are smaller on the average and old platelets larger than those of a normal platelet population. This finding indicates that platelets undergo an aging process.

MEASUREMENT OF THE RADIATION SENSITIVITY OF MOUSE BONE MARROW CELLS BY USE OF I^{131}UdR

Gustavo Cudkowicz D. G. Gosslee[8]
W. B. Stallcup[7] G. M. Shearer
Lou C. Satterfield

Introduction. – The radiosensitivity of mouse bone marrow cells has been measured repeatedly by use of different techniques, all dependent on the facts that (1) marrow cells multiply in the hematopoietic sites of lethally irradiated recipients and (2) the extent of recipient repopulation is a function of the number of transplanted viable stem cells. The present study is an attempt to improve the accuracy of *in vivo* assessment of marrow proliferative capacity and to improve the efficiency of such experiments in terms of the number and duration of observations necessary for reproducible determination of cellular survival kinetics.

Results. – Five days after grafting of syngeneic marrow, spleen repopulation by donor marrow cells was determined in (BALB/c $\times$ A/He) mice exposed to 700 r of x rays. The size of the proliferating donor population was evaluated by measuring the incorporation of iododeoxyuridine-I^{131} (I^{131}UdR), a specific DNA precursor, in the recipient spleen. The relation between I^{131}UdR uptake and the total number of marrow cells transplanted was linear, on a logarithmtic plot, over a range 0.5×10^5 to 16×10^6 normal cells (Fig. 43). Curves identical in slope were obtained with marrow cells exposed *in vitro* to 50, 100, 200, and 400 r of 300-kvp x rays. Within the range over which the cell dose–IUdR response was linear, nine combinations of x-ray exposures and marrow cell doses were selected, according to a "tridimensional" design, and fractions of a single marrow cell suspension were irradiated *in vitro* with graded exposures of x rays and injected into groups of five mice at each combination. The I^{131} UdR uptake-response plane and its linear equation are presented in Fig. 44; the fit of the response plane was not ameliorated by introducing non-linear terms into the equation. The derived survival function is presented in Fig. 45, with the value of the median lethal dose (D_o) and its 95% confidence limits. A second independent experiment faithfully reproduced the survival function, yielding a D_o of 90.5 r with 95% confidence limits of 79.4 to 105.3.

Discussion. – The advantages of using I^{131}UdR-labeling procedures for studying proliferation of marrow cells, as influenced by radiation, are as follows: (1) the recipient mouse does not need to be kept alive for more than five days, (2) I^{131}UdR labeling is specific for DNA of cells in S phase and, therefore, measures marrow cell proliferation *directly*, (3) five observations (five recipient mice) are adequate for reproducible assessment of the

[7]ORINS summer participant, June–August, 1962.

[8]ORNL Mathematics Division.

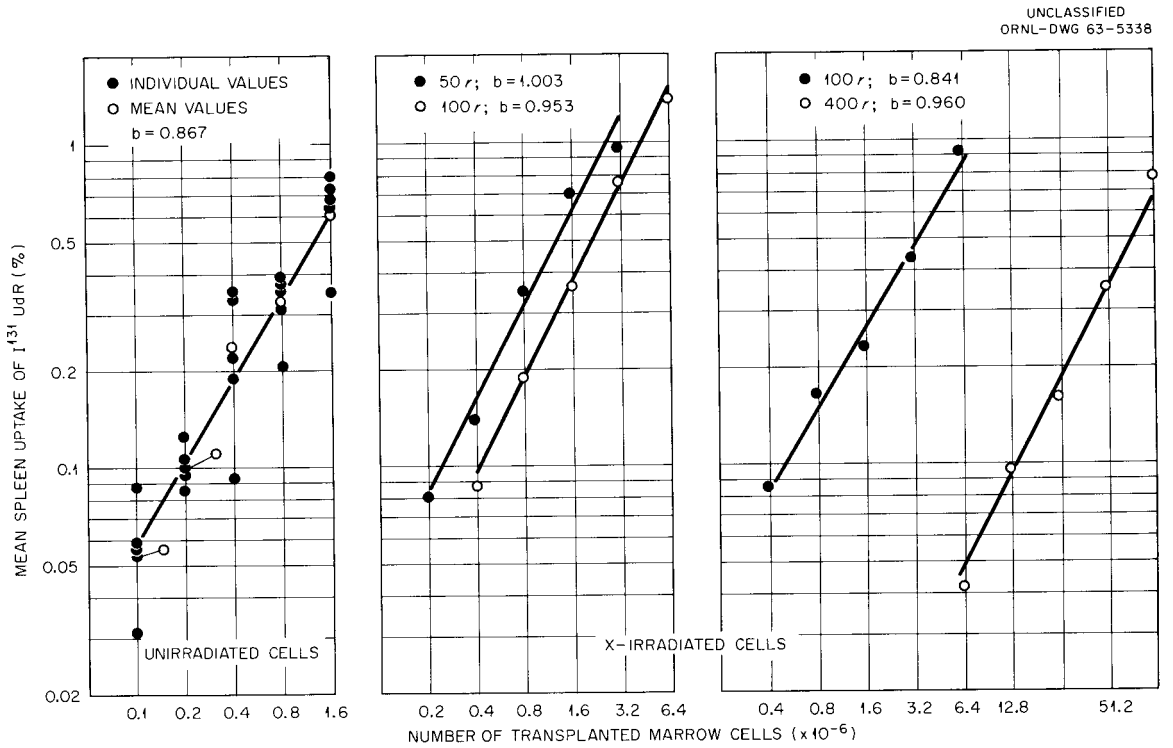

Fig. 43. Splenic Uptake of I^{131}UdR in Relation to the Number of Transplanted Marrow Cells.

proliferative capacity of a given number of irradiated marrow cells. The x-ray survival curves obtained by this method do not display a shoulder, which is consistently obtained in studies in which colony formation by surviving irradiated mammalian cells is determined, although the slopes and D_o values are comparable. The difference in shape between the linear survival curve obtained with this method and the sigmoid curves obtained with other methods may conceivably be attributed to radiation-induced differences in the size of stem cell-derived clones, which are measurable with the I^{131}UdR technique and not with the others.

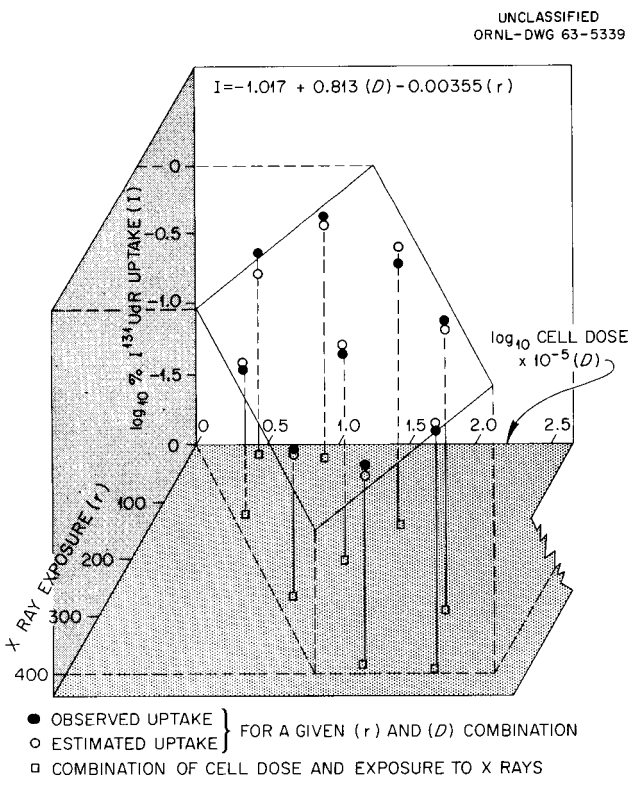

Fig. 44. Splenic Uptake of I^{131}UdR in Relation to the Number of Transplanted Marrow Cells and to X-Ray Exposure.

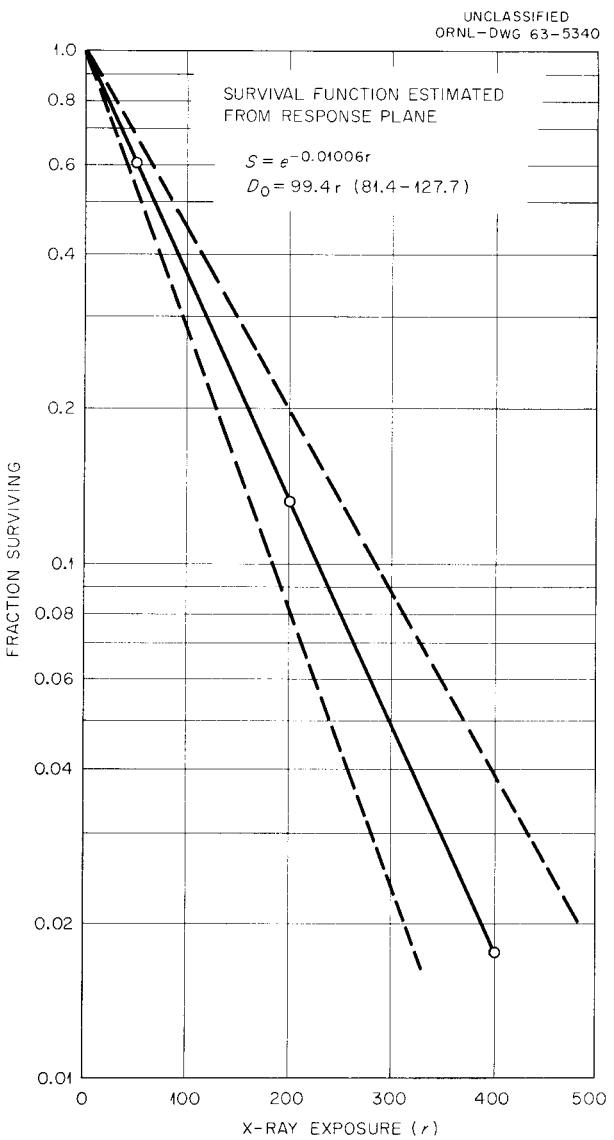

Fig. 45. Survival Function of Marrow Cells Exposed *in Vitro* to X Rays.

GENETIC INTERACTION IN THE INHERITANCE OF GROWTH REQUIREMENTS CONTROLLED BY THE *H-2* LOCUS

Gustavo Cudkowicz
J. H. Stimpfling[9]
Lou C. Satterfield
G. M. Shearer

Introduction. – Earlier work demonstrated that transplanted C57BL/10 marrow cells grew deficiently in F_1 hybrid and in segregating backcross-progeny mice which were *heterozygotes* at the histocompatibility-2 (*H-2*) locus.[10] It was also established that the growth of these cells was optimal (i.e., comparable to that of marrow cells transplanted into syngeneic recipients) in recipients which were $H\text{-}2^b$ homozygotes, in spite of heterozygosity at other *H* loci.[10,11] The present study was undertaken to attempt distinction between the effect of genetic interaction involving the *H-2* locus and the effect of a recessive genetic determinant. Furthermore, several inbred lines of mice were analyzed for deficient-vs-optimal growth of their marrow cells in F_1 hybrids to determine how closely the trait was associated with the $H\text{-}2^b$ allele.

Results. – The growth of transplanted marrow was assessed five days after infusion into x-irradiated recipients by measurement of the uptake of I^{131} UdR in the recipient spleen.[12] C57BL/10 marrow was given to syngeneic F_1 hybrid and to C3H mice irradiated with graded whole-body exposures of x rays. Maximal proliferation of donor cells was observed in syngeneic recipients (Table 32) whenever the x-ray exposure exceeded 600 r. Under these circumstances (in which there was a lack of host-donor histoincompatibility), the splenic uptake of I^{131}UdR depended exclusively on the number of marrow cells injected. C57BL/10 marrow proliferated optimally also in C3H recipients exposed to more than 800 r of x rays; however, growth was suboptimal in the latter recipients at lower exposures. Proliferation of the marrow in hybrid recipients remained suboptimal or absent at all exposure levels, depending on the size of the cell inoculum, although moderate radiosensitivity of the hybrid resistance to C57BL/10 cells was indicated by an increase in uptake of I^{131}UdR in the range 600 to 1000 r.

Several F_1 hybrid mice were produced by crossing individuals of inbred lines, some of them differing at a single *H* locus ("isogenic resistant" lines). One million parental strain marrow cells

[9]Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

[10]G. Cudkowicz *et al.*, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 93.

[11]G. Cudkowicz *et al.*, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p 108.

[12]G. Cudkowicz *et al.*, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 92.

Table 32. Mean Uptake of I^{131}UdR in the Spleen of Syngeneic, Allogeneic, and F_1 Hybrid Mice in Relation to X-Ray Exposure and to Infusion of B/10 Marrow Cells

Number of Donor Cells	Recipient Strain	X-Ray Exposure (r)				
		600	700	800	900	1000
		Mean Uptake per Spleen (% of Injected Dose)[a]				
$\times 10^6$						
1	B/10	0.35 (0.28, 0.45)	0.30 (0.25, 0.37)	0.32 (0.26, 0.39)	0.31 (0.23, 0.42)	0.28 (0.23, 0.35)
1	C3H	0.17 (0.15, 0.20)	0.17 (0.13, 0.21)	0.21 (0.15, 0.31)	0.28 (0.22, 0.37)	0.30 (0.24, 0.38)
1	(B/10 × C3H)F_1	0.02 (0.00, 0.03)	0.02 (0.01, 0.04)		0.01 (0.00, 0.03)	0.05 (0.02, 0.09)
10	(B/10 × C3H)F_1	0.08 (0.07, 0.10)	0.15 (0.08, 0.27)	0.26 (0.20, 0.34)	0.27 (0.15, 0.48)	0.51 (0.39, 0.66)

[a]Seven to nine mice per group; 95% confidence limits in parentheses.

were infused into the hybrids following exposure to 700 r of x rays, and the extent of recipient spleen repopulation was assessed five days later. Whenever parental marrow cells grew deficiently in F_1 hybrids, such cells were grafted also into F_1 hybrids from parents sharing the same *H-2* allele, so that the resulting hybrid would be *H-2* homozygous. The results, presented schematically in Table 33, indicate that the growth of parental marrow cells from $H\text{-}2^b$ lines was generally impaired in *H-2* heterozygotes but unimpaired in *H-2* homozygotes. Substitution or introduction of the $H\text{-}2^b$ allele in "isogenic-resistant" lines correspondingly affected the growth of their marrow cells in F_1 hybrids, except for the line A.BY, in which the $H\text{-}2^b$ allele did not cause deficient marrow growth. The marrow of the lines possessing alleles other than *b* at the *H-2* locus grew without impairment in *H-2* heterozygotes.

Discussion. – The fact that C57BL/10 cells were able to proliferate in C3H mice is contrary to what would be expected if a recessive gene were postulated. The available evidence strongly suggests that the deficient growth of C57BL/10 cells in hybrids is associated with heterozygosity at the *H-2* locus and that some form of interaction involving a subunit of the $H\text{-}2^b$ allele may result in lack of expression of one or more parental strain isoantigens in the F_1 hybrids. The subunit in question can segregate from the other known components of the $H\text{-}2^b$ allele, probably through recombination, as indicated by the growth pattern of A.BY marrow cells.

Table 33. Growth of Transplanted Parental Marrow Cells in *H-2* Homozygous and Heterozygous F_1 Hybrid Mice

Parental Strain	*H-2* Allele	F_1 Recipients[a]	
		H-2 Heterozygotes	$H\text{-}2^b/H\text{-}2^b$
C57BL	b	–	+
B/6	b	–	+
B/10	b	–	+
B/10·BY	b	–	+
B/10·5M	b	–	+
B/10·LP	b	–	+
B/10·D2	d	+	
129	b	–	+
C3H	k	+	
C3H·SW	b	–	+
A	a	+	
A·BY	b	+	+
DBA/2	d	+	
CF	?	+	

[a]+, growth in hybrids as in syngeneic recipients; –, deficient growth in hybrids.

FINGERPRINTS OF POLIOVIRUS TYPE I RNA-P^{32}

R. L. Tyndall K. B. Jacobson[13]
Ernestine Teeter

Introduction. – Previous studies have revealed that fingerprints of Coxsackie B3 virus and/or virus-induced RNA-P^{32} (VVI-RNA) differ markedly from those of the bulk of HeLa cell RNA-P^{32} extractable by hot salt.[14] The VVI RNA-P^{32} in these studies was unmasked by inhibition of HeLa cell RNA synthesis with Actinomycin D. Several investigators[15,16] have shown that poliovirus infection likewise effectively inhibits synthesis of HeLa cell RNA, with subsequent synthesis of poliovirus RNA exclusively. It thus appeared that a fingerprint of RNA-P^{32} synthesized in HeLa cells 2 to 4 hr after poliovirus infection should represent poliovirus RNA-P^{32} *per se* and eliminate the necessity of Actinomycin D. It was also of interest to compare such poliovirus RNA fingerprints with those of Coxsackie B3 VVI RNA.

Results. – One group of 48-hr HeLa cell cultures was overlaid with medium 199. A second group was overlaid with medium 199 containing 1 μg per ml of Actinomycin D and poliovirus type I at a multiplicity of >5.0. The third group of cultures was overlaid with medium 199 and poliovirus type I at a multiplicity of >5.0. After a 30-min preincubation, groups 1 and 2 were inoculated with approximately 50 μc of $P^{32}O_4$ per ml of medium. Group 3 was inoculated with $P^{32}O_4$ after a 2-hr preincubation. All cultures were harvested, and the RNA-P^{32} was extracted with 1 *M* NaCl at 100°C, 4 hr after infection. RNA fingerprints of the three groups were prepared.

Discussion. – Both poliovirus-infected cultures exhibited RNA-P^{32} fingerprints indistinguishable from those of Coxsackie B3 VVI RNA-P^{32} but again were markedly different from those of HeLa cell RNA-P^{32}. The similarity of poliovirus RNA-P^{32} and Coxsackie B3 VVI RNA is not unexpected since both viruses are members of the enterovirus group. Characterization of RNA from other groups of RNA viruses is under study.

[13]Mammalian Cell Regulation (Enzymology) Group.

[14]R. L. Tyndall, K. B. Jacobson, and E. Teeter, in press.

[15]E. F. Zimmerman, M. Heeter, and J. E. Darnell, *Virology* 19, 400 (1963).

[16]M. L. Fenwick, *Virology* 19, 241 (1963).

CHANGES IN PLASMA PROTEINS OF MICE WITH LEUKEMIA

R. C. Allen R. T. Jones

Introduction. – Studies on the plasma proteins in RF/Up male mice with transplanted radiogenic myeloid leukemia have indicated consistent changes, some of which are described in this paper.

Results and Discussion. – Preliminary examination with disk electrophoresis in acrylamide gel has indicated that there is an elevation of two plasma $\beta 1$ globulins. Serial bleeding has suggested that one of the two $\beta 1$ globulins (i.e., the more rapidly migrating one) increases within four days after inoculation of leukemia cells and that the other $\beta 1$ globulin increases during the terminal phase of the disease. Since these proteins migrate electrophoretically in the plasma transferrin region, as determined by Fe^{59} binding studies, the iron-binding capacity of leukemic and normal plasma was studied. It was found that the iron-binding capacity of leukemic plasma was decreased even though the total protein concentration in the transferrin region was increased. Two other protein fractions, which at present may best be described as (1) a low-molecular-weight haptoglobin and (2) an alpha 2 macroglobulin, decrease markedly in the late stages of the disease. In addition, there is a drop in plasma albumin. Changes in lactic acid dehydrogenase (LDH) enzymes which extend those reported earlier have also been noted;[17] that is, with the disk electrophoretic method of zymogram preparation, leukemic plasmas show up to ten LDH bands, whereas normal plasmas have been found to contain only five or six bands. The additional bands found in the leukemic migrate more slowly than those in normal plasma. The origin of the additional bands remains to be determined.

[17]K. Nishio, K. B. Jacobson, and V. K. Jenkins, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1961*, ORNL-3201, pp 94–95.

A GLOMERULOSCLEROSIS IN MICE ASSOCIATED WITH TRANSMITTED MYELOID LEUKEMIA

W. D. Gude V. K. Jenkins

Introduction. – In the study of RF/Up mice bearing serially transmitted radiogenic myeloid leukemia, animals were noted to exhibit glomerulosclerosis. Consequently, this survey was carried out to investigate the incidence of the kidney lesion in relation to the development of the neoplasm.

Results and Discussion. – In contrast to the relative infrequency and mildness of glomerular changes in controls and nonleukemic recipients of passage materials, glomerular lesions were frequent and severe in leukemic mice (Table 34). Although these changes superficially resembled those observed to occur spontaneously in aging RF mice,[18] their frequency and severity greatly exceeded the natural level for control mice of comparable age. Hence, they may have resulted from inoculation of leukemic passage materials. Their absence in inoculated mice that failed to develop leukemia suggests that their induction depended on the neoplasm itself, but this interpretation, their time of onset relative to the appearance of leukemia, and other aspects of their pathogenesis remain to be established.

[18] W. D. Gude and A. C. Upton. *Am. J. Pathol.* **40**, 699 (1962).

Table 34. Glomerular Lesions in Relation to the Inoculation of Leukemic Passage Materials and the Subsequent Development of Leukemia

Passage Material Injected	Number of Mice	Age at Sacrifice (months)	Leukemia	Severity of Glomerular Change[a]				
				Negative	±	+	++	+++
None (control)	52	1–5	Absent	33	11	8	0	0
Leukemia cells	7	2–3	Absent	5	2			
Leukemia ultracentrifugate	5	5	Absent	4	1			
Leukemia cells	12	2–4	Present		1	4	6	1
Leukemia ultracentrifugate	27	1–6	Present		2	7	13	5
Leukemia filtrate	11	1–6	Present		2	6	3	
Plasma ultracentrifugate	7	1–4	Present			1	4	2

[a] Figures denote numbers of mice showing each of the various degrees of glomerular change, as scored by histological criteria described earlier [W. D. Gude and A. C. Upton, *Am. J. Physiol.* **40**, 699 (1962)].

Cell Physiology

N. G. Anderson	
Subcellular Physiology N. G. Anderson G. M. Padilla C. L. Burger[a] B. T. Cole[b] E. C. Horn[b] T. W. James[c] W. T. Lammers[d] C. A. Price[b] Herbert Schuel[c] S. R. Tipton[b] P. A. Van Dreal[c] R. E. Canning R. Elizabeth Lybarger C. T. Rankin, Jr. Mary L. Anderson[b] G. B. Cline[d] Regina Schubel[b]	**Cell Biophysics** Peter Mazur J. G. Green[c] S. P. Leibo[e] E. Jane Holloway **Molecular Physiology** W. D. Fisher A. W. Prestayko[f]
[a]Research participant. [b]Consultant. [c]USPHS Fellow. [d]Loanee. [e]Research associate. [f]Visiting investigator from abroad.	

THE JOINT NIH-AEC ZONAL CENTRIFUGE DEVELOPMENT PROGRAM

N. G. Anderson C. L. Burger

Introduction. – The development of basic new tools requires close collaboration between specialists in both the biological and physical sciences. Initially, it is essential to develop the (1) rotors and drive systems, (2) seals, and (3) containment systems necessary for work with virus materials. Three types of low-speed (A series) rotors have been developed. The stability problems which limited the B-II rotor to approximately 25,000 rpm have now been solved, and routine operation at 40,000 rpm (90,800 × *g*) has been obtained using a new experimental upper damping bearing. In addition, high-speed zonal ultracentrifuges (C-II to C-V, 141,000 rpm at 310,000 × *g* to 40,000 rpm at 91,000 × *g*) have been designed and tested by the personnel at the Oak Ridge Gaseous Diffusion Plant (ORGDP). These rotors are in various stages of development, as noted in a recent report.[1] A prototype of an ultrahigh-speed centrifugal system (D series, capable of 1.2 million × *g*) has been tested to 200,000 rpm.

[1] *Joint NIH–AEC Zonal Centrifuge Development Program Semiann. Rept. January 1–June 30, 1963*, ORNL-3502 (Special).

This work, initiated in the ORNL Biology Division, has required the talents of various groups at ORGDP. Pertinent theoretical studies have been undertaken in many areas, some of which are (1) rotor stability and design, including fabrication; (2) metallurgical studies, including stress analysis and corrosion resistance; (3) instrumentation and controls; (4) chemistry of cesium chloride for reprocessing; (5) electron microscopy; (6) mathematical analyses of flow rates for continuous centrifugation; (7) containment and sterilization; and (8) data reduction.

Under the program the principles of zonal centrifugation developed in the Biology Division have been combined with certain revolutionary advances made by the ORGDP Technical Division in high-speed centrifugation and rotor fabrication. The aim is to perfect high-resolution fractionation methods for separating particles ranging in size from whole cells to protein molecules. Virus particles fall within this range, and to date two types of adenovirus and respiratory syncytial virus have been separated and concentrated using the B and C series rotors. The biological implications of these centrifuge systems extend from determining the distribution of drugs or other materials in cells to the separating of viruses from tumors. Also under study is the possibility of using zonal centrifuges for vaccine production. These problems are discussed in detail elsewhere.[1]

Ancillary studies carried out by ORGDP have included (1) pressure filtration and dialysis; (2) ultrahigh-pressure chromatography; (3) flame ionization analysis, capable of resolving 10^{-10} g of carbon; and (4) cell synchronization (in which 10 to 60 liters of cells may be continuously grown).

Other biological studies include a new method for cesium chloride banding using angle-head rotors, cell synchronization studies, sugar analyzer development, automated nucleotide studies, separation of radionuclides from river water, distribution of particulates in *Escherichia coli*, and protein synthesis on ribosomes from *E. coli* and rat liver thymus.

The ultimate aim of this program is to develop methods for viewing the cell as a spectrum of particles, each portion of which may be analyzed biochemically and biophysically. Three general areas of investigation are included in this broad program and are closely interrelated in the achievement of the final goals.

1. For studies on subcellular particles it is important to have available a population of cells which are as nearly identical in size, composition, and physiological state as possible. Studies on mass cell synchronization, therefore, will in the future occupy an increasing portion of the total effort.

2. Development of centrifugal methods for subcellular particles, which constitutes the bulk of the present effort and will continue so to do for two or three years, will eventually be decreased to a supporting effort.

3. The development of molecular separation methods, which has been a minor effort in the form of the automated nucleotide and carbohydrate analytical systems, will be gradually increased as high-pressure chromatography and new on-stream analytical systems are developed.

The biological studies undertaken in parallel with the developmental effort are (1) isolation of virus particles from complex mixtures, (2) examination of changes in cell composition which follow virus infection, and (3) alterations in cell composition during the cell cycle. In collaboration with interested groups outside the Laboratory, studies are being undertaken on the intracellular localization of drugs, herbicides, and pesticides, since the systems described are readily adaptable to such problems.

ISOPYCNIC SEPARATIONS OF PARTICLES PREVIOUSLY ISOLATED BY RATE-ZONAL CENTRIFUGATION

N. G. Anderson W. D. Fisher
C. T. Rankin, Jr.

Introduction. – The fractions collected from the zonal ultracentrifuge that are suspected of containing virus particles must now be separated on the basis of particle density by banding in cesium chloride or other dense solution. The centrifugal systems that will ultimately be used for this cannot be visualized with certainty at this time. Centrifugation of particles from sucrose solutions into small-volume CsCl gradients, for example, may require specially designed tubes and rotors. At least 12, and preferably many more, samples must be examined at one time. Methods have

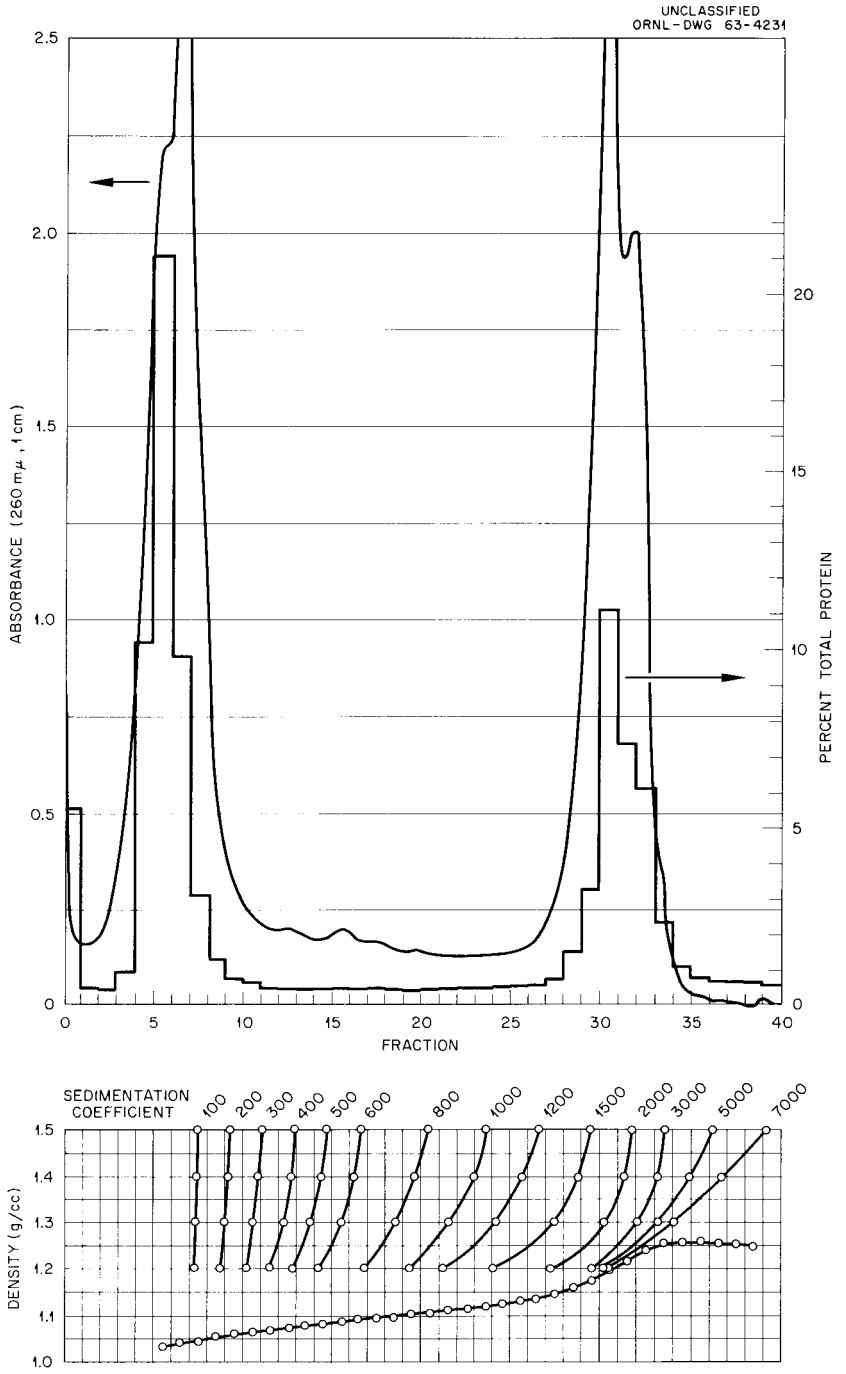

Fig. 46. Fractionation of Rat Spleen Brei in B-II Zonal Centrifuge. A total of 3.42 g of spleen from four adult Sprague-Dawley rats was homogenized in 8% sucrose to give a total volume of 24 ml. One ml of T3 phage solution containing 4×10^{12} phage particles was added. The 1000-ml sucrose gradient varied linearly with volume from 10 to 30% sucrose, with a cushion of 55% sucrose. An overlay of 216 ml of sodium phosphate NaCl (pH 7.5, $\mu = 0.1$) was used, and the homogenate was centrifuged 1 hr at 20,000 rpm. The value of $\omega^2 t$ at the start of unloading was $16{,}912 \times 10^6$. Protein was determined using an automated Folin biuret method and was expressed as percent of total protein in the starting sample. Sedimentation coefficients plotted in terms of particle density are plotted below the chart.

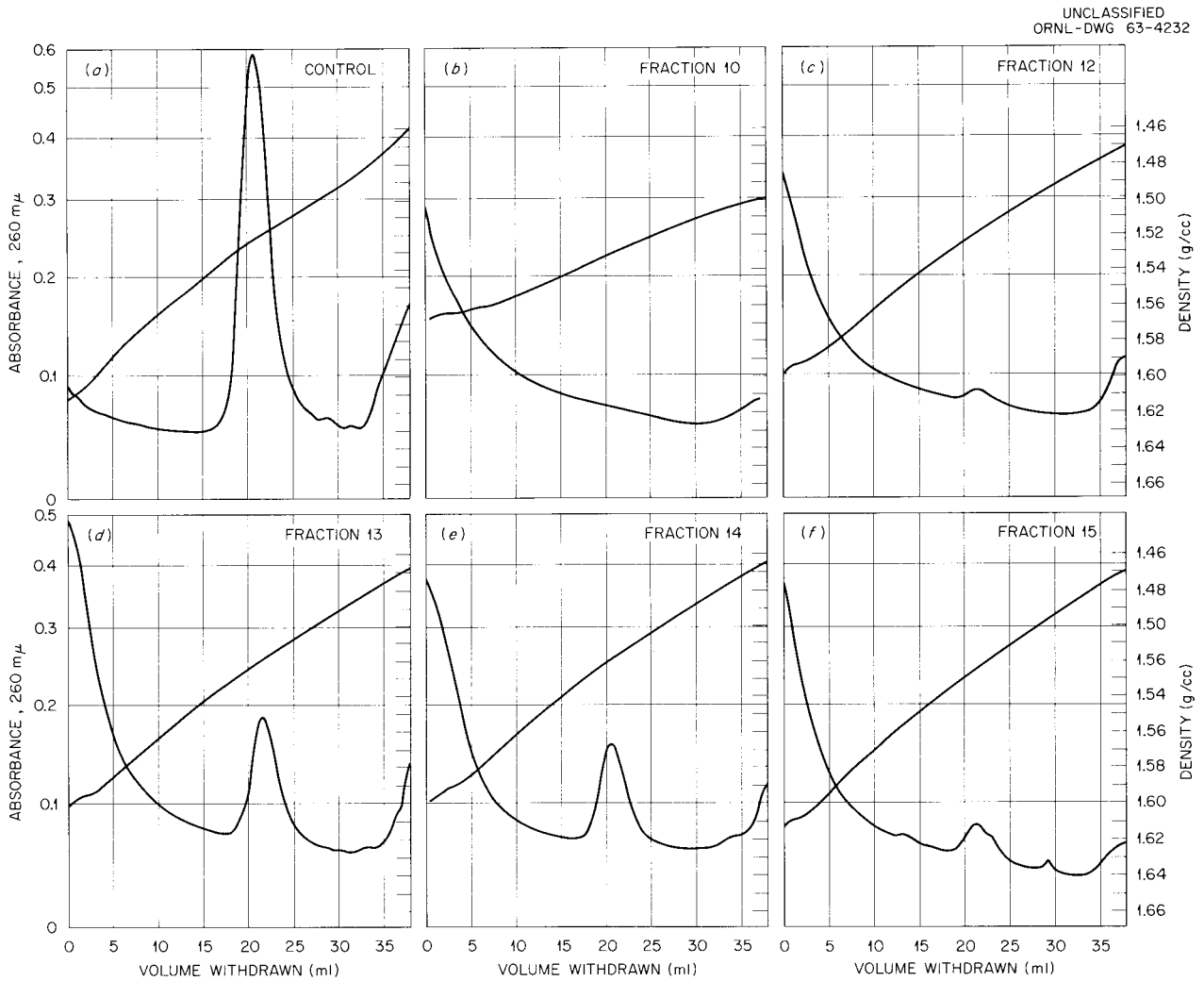

Fig. 47. Banding of T3 Phage in CsCl. In (*a*), the same number of phage was added to the spleen brei described in Fig. 46 and was similarly centrifuged. In (*b*)–(*f*), five fractions from the rate-zonal run shown in Fig. 46 have been banded in CsCl, and the runs have been monitored using the refractive index–absorbance monitor described in a subsequent section. The majority of the T3 virus was found in fractions 13 and 14. Very little background material having the same density as T3 was found in tube 10, a result indicating the small amount of contaminating material found in this sedimentation rate region.

been devised for equilibrium banding of virus particles in angle-head rotors and for automatically monitoring the gradients for refractive index and absorbance.[1]

Experimental. – To explore rate-zonal centrifugation in the B-II rotor, followed by CsCl banding of the virus-containing fractions, T3 phage particles were added to a rat spleen brei, which was then centrifuged (Fig. 46). As observed in the B-II rotor uv monitor, the virus peak is small. Presence of virus in this peak was confirmed by electron microscopy. A number of fractions were then centrifuged for 2 hr at 30,000 rpm in the Spinco No. 30 rotor, and the pellets were resuspended in CsCl (density 1.5). After centrifugation for 15 hr at 24,000 rpm, the virus was recovered (Fig. 47). Although the virus sample represents a very large number of particles (4×10^{12}), the virus mass was only 560 μg, 0.0163% of the sample mass. The phage particles were easily

identified in the electron microscope, and only a small amount of contamination was observed. The T3 sample was equivalent to approximately 6000 particles per cell – a concentration yielding about 10 particles per 250-A section of a cell. It is evident that the method can be scaled down by nearly a full order of magnitude using available methods and can possibly be increased in sensitivity by two orders of magnitude using more refined methods for scanning isopycnic gradients. Upon scanning the gradients with the electron microscope, a considerable additional sensitivity can be obtained.

Discussion. – We wish to ultimately scan human tumors and other tissues for virus particles. To properly interpret the results, it will be necessary to make extensive observations on "normal" materials to discover the nature of any subcellular particles that may resemble viruses in physical properties. Preliminary results obtained with human leukemic plasma show the presence of particles having the size, morphology, and density of viruses.

DETECTION OF VIRUS PARTICLES IN GRADIENTS

N. G. Anderson

Introduction. – In the detection of virus particles by ultraviolet (uv) absorption, the lower limit with present systems is approximately 0.1 absorbance unit. With a small virus such as polio, this represents approximately 1.3×10^{12} particles; with a large one such as vaccinia, the same absorbancy is produced by about 1.4×10^{10} physical particles. If one particle per cell is to be detected, then the number of original cells must equal the number of particles.

For example, in rat liver 1 g of tissue contains (2 to 4) $\times 10^8$ cells. The upper limit for tissue used directly in the B-II rotor is about 10 g, giving about 3×10^9 cells. This is one order of magnitude below the uv absorption limits for vaccinia, and three below that for polio.

However, if 10^8 virus particles can be isolated from 1 g of tissue free of other contamination and in a volume of 1 ml, then their detection through conventional counting techniques by electron microscopy is fairly simple. Further, if the isopycnic separations are done in a small volume, the limit of 0.1 absorbance unit may be considerably decreased.

For present purposes the osmotic properties of subcellular particles will be ignored, and the question that will be asked is: What percentage of the protein in a tissue sediments in a sucrose density gradient under a given set of conditions to a position that would also have been reached by a virus of known properties? The amount of such "contamination" will affect directly the level at which virus particles may be detected in the electron microscope.

Experimental. – The results of typical experiments are shown in Figs. 47 and 48. The amount of protein present was determined by an automated method. In rat liver approximately 0.5 to 1.5% of the total cell protein is found in each 40-ml fraction in the area between the soluble phase and the mitochondrial peak. Most of this material is probably fragmented endoplasmic reticulum. This conclusion is supported by the findings obtained with rat spleen, shown in Fig. 46. In most tumor cells and in many cell lines used in tissue culture, the amount of endoplasmic reticulum is less than that in liver. If a virus particle is restricted to only a few of the 40-ml fractions, it is evident that considerable purification is achieved in one zonal centrifuge run.

Discussion. – These studies do not demonstrate the full capabilities of the B-II rotor system, however. To retain the larger particulates in the rotor, a short, steep gradient was used. In practice, nuclei, cells, and mitochondria can be sedimented to the rotor wall or to a "cushion" near the rotor edge, and the viruslike particle range can be extended considerably.

It is also evident that preconcentration can be used to increase greatly the amount of virus-range material in the rotor. Thus the large particles may be sedimented at low speed, and the virus-sized material may be sedimented at higher speed, resuspended, and then run in the zonal ultracentrifuge. As much as 100 g of tissue or cells may be processed in this manner. However, in the present work, efforts have been made to develop general methods that would not necessarily involve pelleting virus materials at any stage, so that very sensitive materials may be isolated.

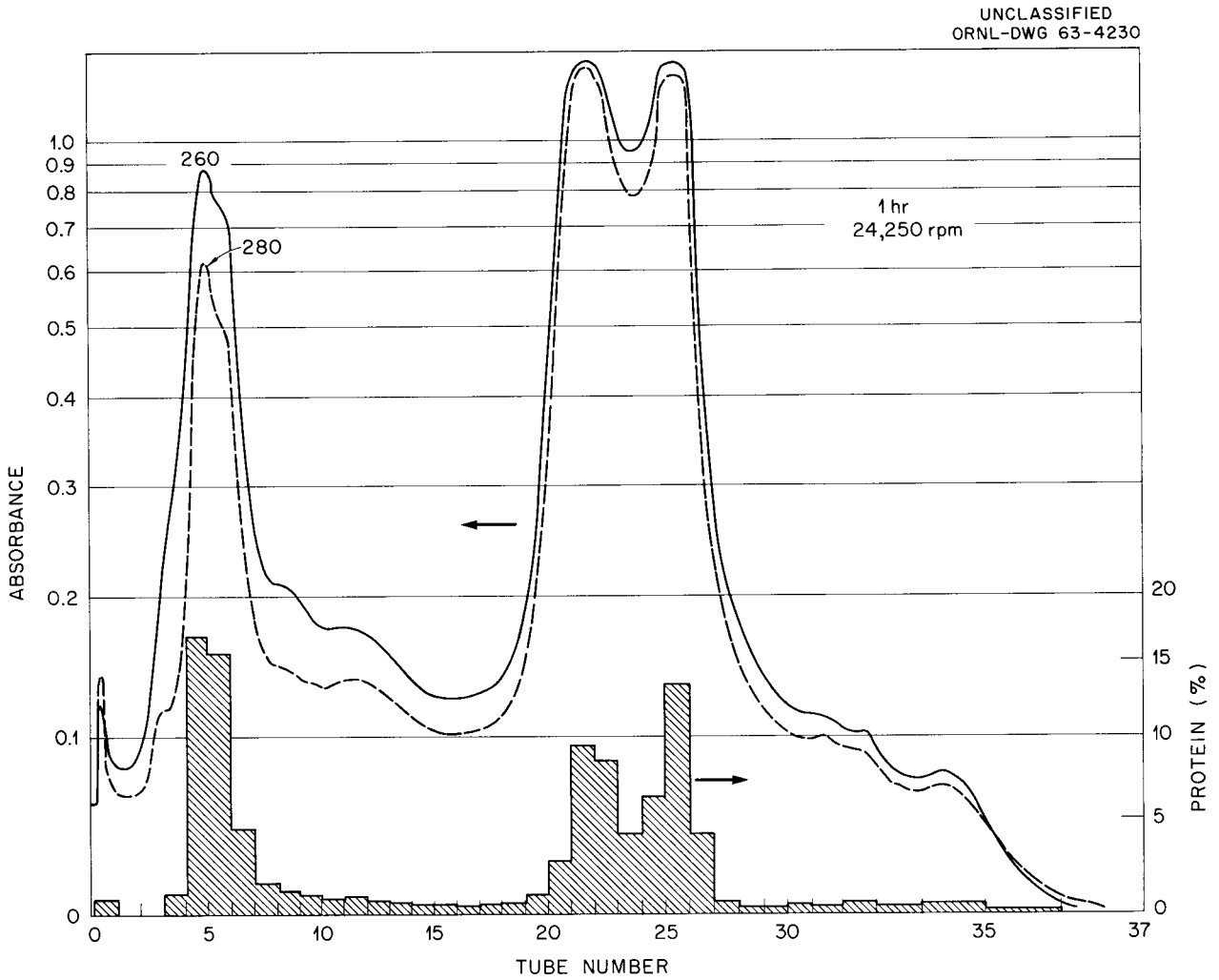

Fig. 48. Distribution of Absorbance and Protein in Rat Liver Brei Centrifuged in Sucrose Density Gradient in B-II Zonal Centrifuge. A total of 12.5 ml of a 20% perfused rat liver brei was introduced centripetal to a 1200-ml 17 to 55% sucrose gradient with a cushion of 66% sucrose. An overlay of 216 ml of Miller Golder buffer (pH 7.5, μ = 0.1) was applied, and the homogenate was centrifuged 1 hr at 24,250 rpm at 5°C. Protein is shown as percent of the total sample found in each fraction collected.

LARGE-SCALE VIRUS ISOLATION

N. G. Anderson C. T. Rankin, Jr.
R. E. Canning C. L. Burger
W. D. Fisher

Introduction. – Although stable spherical viruses are excellent test objects for studying the performance of zonal centrifuges, many of the most important isolation problems involve virus particles that are unstable and do not necessarily behave ideally. It is important, therefore, to determine the applicability of zonal centrifuges to the isolation of a number of different types of viruses.

Initially, four viruses presenting quite different isolation problems have been considered. These are adenovirus 2, Echo 28, the respiratory syncytial virus, and the Rauscher mouse leukemia virus. The first is a nuclear virus that must be separated from other subcellular particles. Echo 28 is a small virus growing to very low titer in tissue culture. The respiratory virus is a very sensitive one that may require the development

of rapid, refined concentration methods; while the Rauscher virus may serve as a prototype for the oncogenic viruses now being sought in human materials.

The basic problems are (1) concentration of the virus into a small volume, (2) separation based on sedimentation rate, and (3) sedimentation based on particle density. For some applications the B-II rotor system[2] serves all three purposes. However, for very small virus particles this rotor has not provided sufficient centrifugal force at a reasonable flow rate. Further, a number of viruses lose infectivity when sedimented against a solid surface. Alternate approaches to the concentration of virus particles, therefore, have been investigated. These include ultrafiltration and sedimentation in a large-capacity rotor (e.g. C-V) directly into a dense medium such as a CsCl solution. For separations based on rate the rotors B-II and the newer modification (B-V) will probably continue to be most useful. To achieve purification by isopycnic banding, a series of rotors (C-II to C-IV) are being developed for samples of varying size, while angle-head centrifugation of CsCl-suspended particles has been developed where many different individual samples are to be banded. As these methods are perfected, they will be applied to the isolation of the viruses listed.

Experimental. – The initial studies on the isolation of adenovirus 2 were done on whole cultures (cells plus culture fluid) that had been frozen and thawed several times to release the virus particles. The fluid was passed through the B-II rotor, using it as a continuous flow centrifuge. The sedimented virus was then displaced off the wall and banded in a CsCl density gradient. Because of instabilities observed at high speeds, the rotor was operated at only 20,000 rpm, a speed insufficient to achieve complete sedimentation of the virus. Although encouraging concentrations were obtained, further study on continuous-flow separation of adenoviruses has been delayed until higher speeds can be routinely achieved.

The second method investigated involved preconcentration of the culture fluid lysate by dialysis against high-molecular-weight Dextran. The concentrated material (53 ml) was then separated by rate-zonal centrifugation through a sucrose density gradient for 30 min at 20,000 rpm. The results are shown in Fig. 49. The virus was found in the sharp peak at the 1.234 density level using the electron microscope. Whether this is the isopycnic point for the virus in sucrose remains to be seen. Infectivity studies by Maurice Mufson at the Laboratory of Infectious Diseases at the National Institutes of Health indicate that the majority of the virus particles are in this peak, with a few being scattered in the lighter portions of the gradient. The peak material was banded in CsCl in an angle-head rotor. A photomicrograph of purified material is shown in Fig. 50.

Discussion. – The results shown in Fig. 49 are surprising because the virus sedimented much farther down the gradient than would be expected on the basis of physical dimensions of the particles. This strongly suggests that the virus particles may aggregate in solution. Preliminary studies carried out by W. W. Harris, in which adenovirus 2 was sedimented onto a flat surface from solutions containing varying concentrations of virus, suggest that the degree of aggregation is concentration-dependent. The peak observed to the right in Fig. 49 may therefore represent highly aggregated material, while the small amount of infectivity observed closer to the starting zone is probably due to the presence of aggregates of smaller size.

Studies on the pH dependence of aggregation are now being done so that rate-zonal centrifugation may be used to purify adenoviruses.

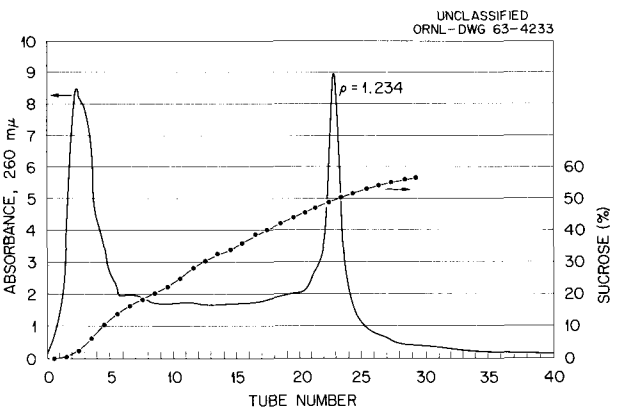

Fig. 49. Separation of Soluble Material and Adenovirus 2 in Sucrose Gradient in the B-II Rotor.

[2] *Joint NIH–AEC Centrifuge Development Program Semiann. Rept. July 1 – December 31, 1962*, ORNL-3415 (Special).

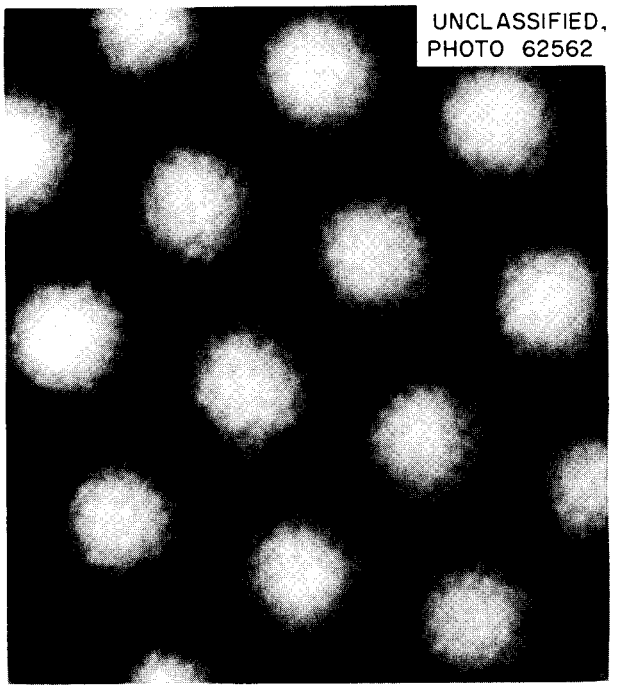

Fig. 50. Photomicrograph of Purified Adenovirus 2. 320,000 X.

RATE-ZONAL SEPARATION OF LARGE PARTICULATES FROM BACTERIAL PROTOPLASTS

G. B. Cline W. D. Fisher

Introduction. – Rate-zonal separation in sucrose gradients in the B-II or B-IV rotor systems[3,4] of water lysates of *Escherichia coli* protoplasts reveals a component in the leading heavy peak which contains particles of ribosomal dimensions. Since aggregated ribosomes have been found to be the site of protein synthesis in rabbit reticulocytes,[5] it seemed of interest to analyze the components of the leading peak more critically to determine whether or not aggregated ribosomal clumps were present in *E. coli* which could be shown to be active in protein synthesis.

The relation between the ordering of the ribosomes on the surface of membranes and protein synthesis deserves careful attention since the organization of ribosomes into polysomes has been shown to be important in other systems. In the *E. coli* materials prepared by methods involving high shearing fields, DNA molecules are broken and complex ordered macromolecular structures would be disrupted. It is essential, therefore, to develop extremely gentle methods for disassembling cell structures which would preserve as many of the relationships found in the intact cell as possible. To determine whether the ordering of ribosomes on the cell membrane is characteristic only of β-galactosidase-induced protoplasts, *E. coli* infected with T2 bacteriophage have also been studied. After lysis the phage and large particulate material were separated from the free ribosomes and soluble substances by zonal centrifugation under conditions identical to those shown in Fig. 51. An electron micrograph showing organized ribosomes in gradient fraction 33 is shown in Fig. 52. These structures, therefore, are not characteristic only of β-galactosidase-induced cells.

Results and Discussion. – The results of several experiments using the analytical ultracentrifuge show that there are particulates in the water lysate of protoplasts which have sedimentation coefficients ranging from 150-S to 1200-S and have a 280/260 mμ ratio of 0.6. Lysates of protoplasts induced for β-galactosidase show that the enzyme distribution is coincident with both the soluble as well as the leading peak (Fig. 51).

Low-resolution electron micrographs of the leading peak show particles containing organized subunits as well as protoplast ghosts and so-called "lipid granules."[6]

Since the higher levels of cell organization may be important in the control of synthetic activities, it is important to attempt the isolation of the fragments showing the organized ribosomal arrays. Fractionation of both the leading heavy fraction and the leading edge of the soluble peak observed from protoplast lysates is being explored by rate-zonal centrifugation and isopycnic banding in CsCl using new methods for CsCl centrifugation.

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[3] N. G. Anderson and C. L. Burger, *Science* **136**, 646 (1962).

[4] N. G. Anderson, *J. Phys. Chem.* **66**, 1984 (1962).

[5] P. A. Marks, E. R. Burks, and D. Schlessinger, *Proc. Natl. Acad. Sci. U.S.* **48**, 2163 (1962).

[6] C. Weibull, *J. Bacteriol.* **66**, 696 (1953).

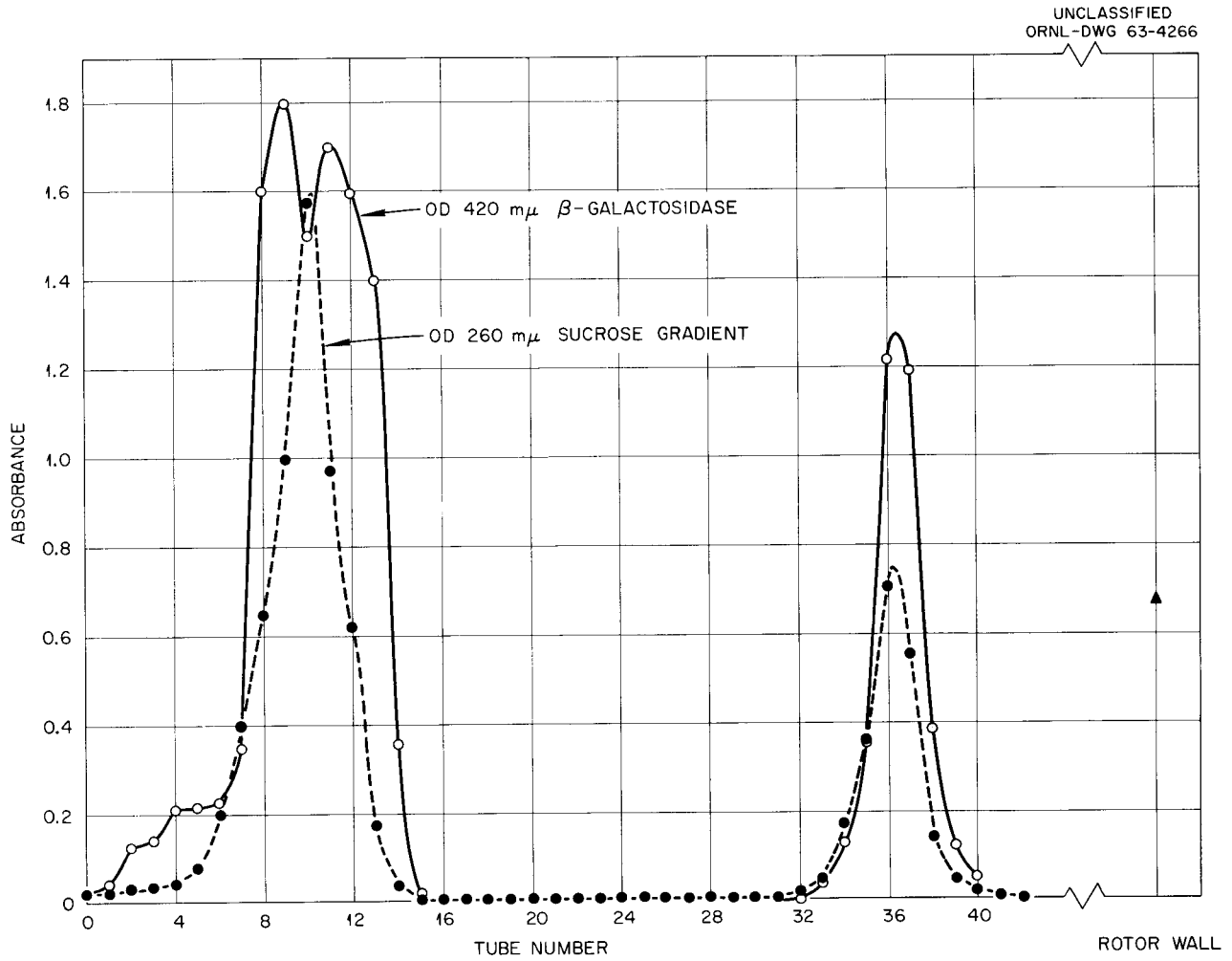

Fig. 51. Separation of *E. coli* B Protoplast Lysate Particles in the B-V Zonal Ultracentrifuge Rotor.

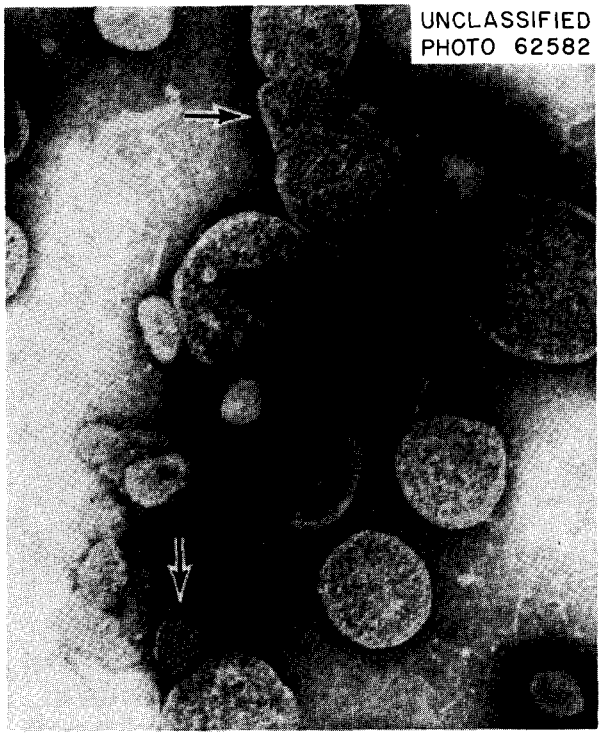

Fig. 52. Particles Containing Organized Ribosomes in Lysates of T2-Infected *E. coli*. Negatively stained with phosphotungstate.

PROTEIN SYNTHESIS ON RIBOSOMES

G. B. Cline A. W. Prestayko W. D. Fisher

Introduction. – The B-II and B-IV rotor systems, when operated at their maximum speed of 40,000 rpm, are designed to separate quantities of ribosomes and ribosomal subunits from cell extracts in $1\frac{1}{2}$ to 2 hr of centrifugation. In a previous report[2] the separation of ribosomes which had been previously separated from the majority of soluble materials of the cell by preparative ultracentrifugation was demonstrated. Prepacking of ribosomes lengthens the time interval between initial cell disruption and final separation of the particles in a density gradient and is known to alter the physical properties of the particles.

It is essential to know whether or not rapidly prepared, broken cell suspensions may be introduced directly into the B-II or B-IV rotors and whether or not the amount of material which may be introduced in a narrow starting zone will be sufficient to observe on an ultraviolet run monitor and for subsequent biochemical analysis. If rapid separation of ribosomal particulates from the original homogenate can be achieved, the artifacts associated with presedimentation and prolonged incubation may be minimized. More important, pulse-labeling experiments to follow protein synthesis on these subcellular particles may be initiated. The experiments reported here were performed before the new B-II and B-IV upper bearing systems (which make possible operation to 40,000 rpm) became available.

Results and Discussion. – The work described here is part of a program to investigate the role of ribosomal particles isolated by zonal ultracentrifugation in protein synthesis. Ribosomes from log phase cultures of *Escherichia coli* and from isolated rat thymus nuclei have been separated in sucrose density gradients, as shown in Figs. 53 and 54. The *E. coli* cells were induced for β-*d*-galactosidase, disrupted in a constant-pressure French press at 14,000 psi, and loaded into the B-IV rotor at 5°C. Figure 53 shows the absorbance of the effluent gradient stream at 260, 280, and 415 mμ and the distribution of β-galactosidase. This demonstrates the feasibility of such direct separations. Pulse-labeling studies of induced enzyme synthesis are in progress. Figure 54 shows the uv run monitor chart for the rat thymus nuclear ribosomes. The S_{20} values were extrapolated from comparable analytical ultracentrifuge runs since the computer program for the determination of sedimentation coefficients with the B-IV rotor is not complete. *In vitro* studies on the composition and enzyme complement of nuclear ribosomes and their protein-synthesizing capabilities are under way.

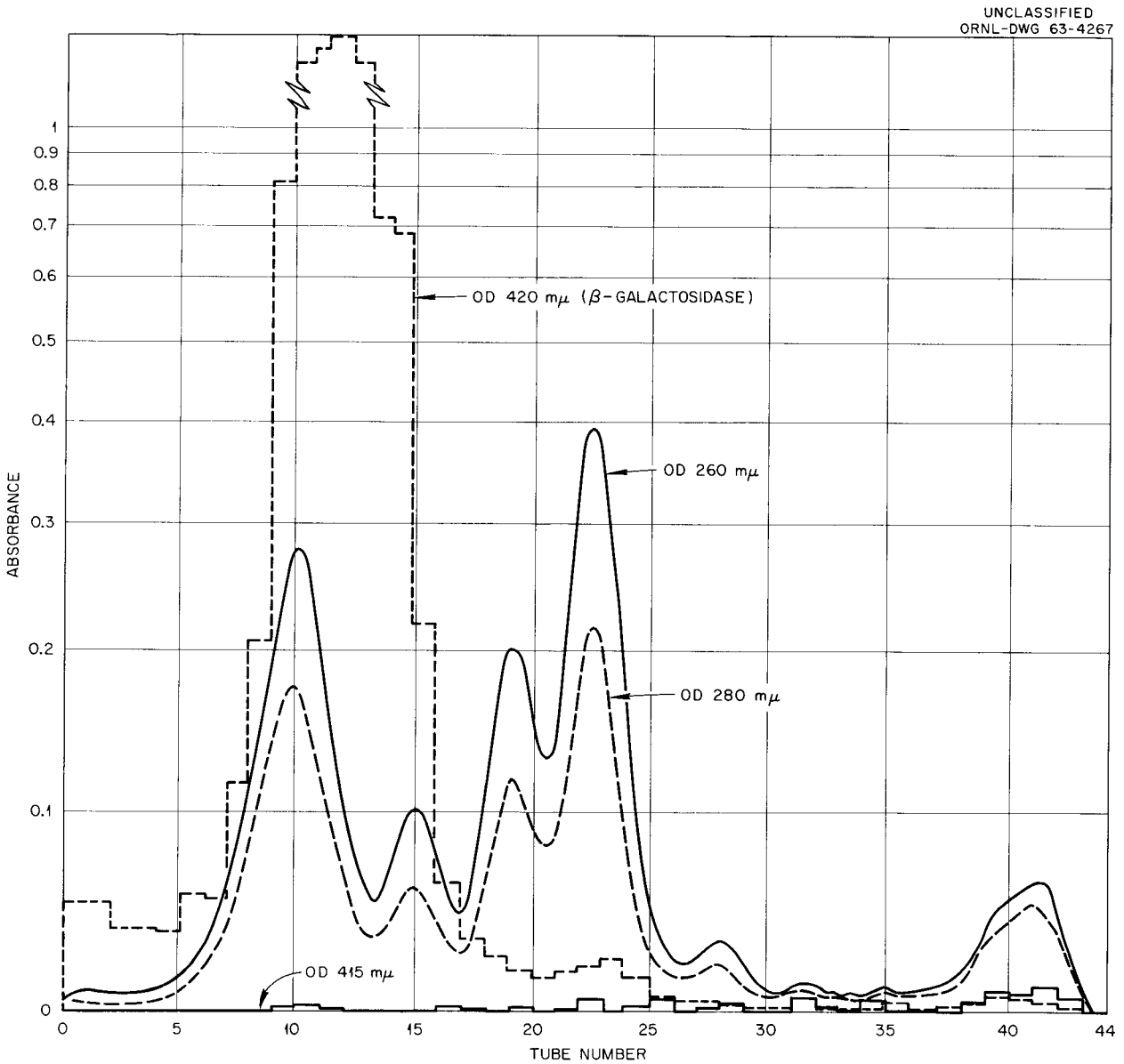

Fig. 53. Separation of Ribosomes from β-Galactosidase-Induced *E. coli*. Absorbance at 260 mμ was determined by the centrifuge run monitor, 280 and 415 mμ absorbancies being obtained from the collected fractions. The β-galactosidase was determined using *o*-nitrophenyl-*d*-galactopyranoside as the substrate. The rotor was loaded and unloaded at 3000 rpm and run for 5 hr at 25,000 rpm. The sucrose density gradient extended from 10 to 30%, with a 55% (wt/wt) cushion.

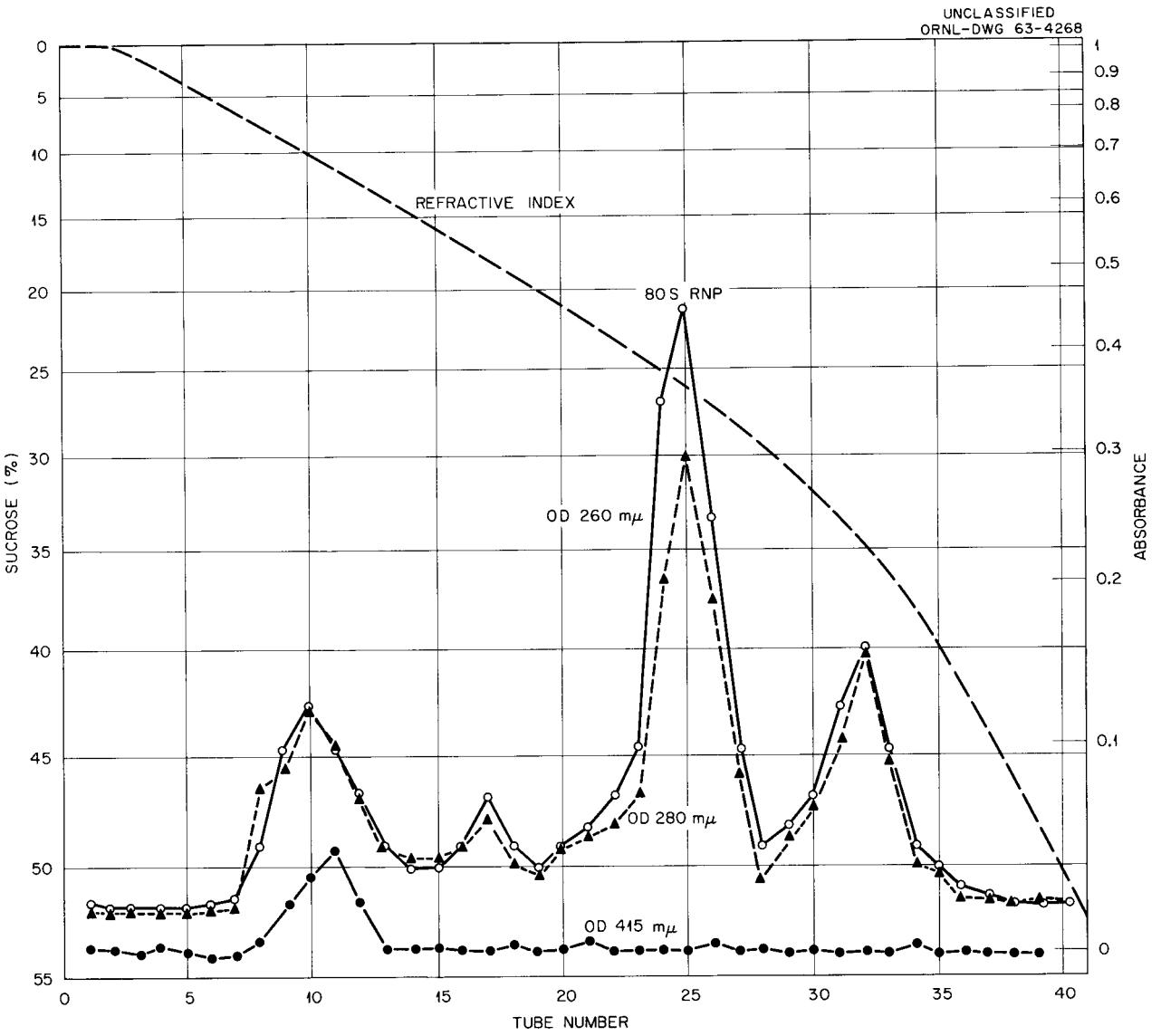

Fig. 54. Separation of Nuclear Ribosomes from 5 g of Isolated Rat Thymus Nuclei in the B-V Rotor in a 10 to 30% Sucrose Gradient. Plots at 260, 280, and 415 mμ show absorbance of rotor effluent stream. The refractive index was automatically recorded. Loading and unloading done at 3000 rpm, while the separation was done at 25,000 rpm for 4 hr.

STUDIES ON SYNCHRONIZED CELL DIVISION

G. M. Padilla T. W. James P. A. Van Dreal

Introduction. – The randomness of individual cell divisions that is seen in a growing cell population introduces a lack of temporal definition to the causal link that may exist between a given physiological process and mitosis. This difficulty is in part avoided if single cells are the experimental material. Snychronization of cell division helps one study this link, partly because the development of a synchrony-inducing technique involves the critical examination of how the cell cycle is affected. In cases where a high degree of synchronization is achieved, the resultant population of cells becomes a model of

the single cell; but, unlike single cell studies, large quantities of material are available for further analysis and experimentation. At the same time a particular physiological event occurring throughout the entire culture may be examined in relation to any given phase of the cell cycle.

The experimental cell synchronizer was described in a previous report.[7] This unit consists of a 5-liter water-jacketed culture vessel programmed with a repetitive temperature cycle (17.5 hr at 14.5°C and 6.5 hr at 28.5°C) which induces cell division synchrony in the colorless flagellate *Astasia longa*.[8,9] A continuous cell-density monitor has also been developed. With potentiometric electrodes (e.g., Beckman oxygen electrode) and the synchronizer and density monitor, one may now determine continuously the pattern of respiratory activity of synchronized cells. The results of these investigations are presented in this report. In addition, the results of initial investigations on the role of chemical constituents of the medium in the induction of synchrony and the effects of low-level radiation throughout the cell cycle are outlined.

Respiratory Activity of Synchronized Cells. – The initial process that is being examined as a function of the synchronized cell cycle is the respiratory activity. Similar studies have shown that fluctuations exist in the oxygen consumption of synchronized cells.[10] The present investigations are based on the continuous measurement of oxygen tensions with oxygen electrodes that retain their potentiometric function for considerable periods of time. Figure 55 illustrates a typical experiment in which changes in the oxygen tension in the medium of synchronously dividing *Astasia* have been continuously recorded. Three traces are shown: (a) the temperature record; (b) the oxygen tension (pO_2), expressed in mm Hg for the dissolved oxygen; and (c) the population density, expressed in cell numbers per milliliter of culture.

During the cold period (14.5°C) the oxygen tension decreases very slowly from approximately 95 mm Hg to 65 mm Hg pO_2. During this interval the cell density remains virtually constant at about 120,000 cells/ml. As soon as the warm period begins (28.5°C), there is a rapid decrease in the level of dissolved oxygen to a partial pressure of 7 mm Hg. This rapid decrease, reflecting an increased oxygen consumption by the cells, occurs in the predivision period, which is coincident with initial phases of mitosis.[9] A slight decrease occurs throughout the burst of division, but at this analyzer setting it is hardly noticeable.

On the basis of the following formulation,

$$\frac{dC}{dt} = \frac{dD}{dt} - \frac{dO_2}{dt},$$

relating the apparent rate of oxygen utilization by the entire culture (dC/dt) to the rate of diffusion of oxygen (dD/dt) into a culture of a given volume, stirring rate, and at a given temperature, oxygen tension, etc., one may derive the rate of oxygen utilization (dO_2/dt) by the cells at any time. In essence, one is treating the entire culture as a large-scale respirometer, differing from the conventional type in that the cells respire *ad libitum* and are permitted to grow.

Figure 56 shows the data derived in this manner. It is clear that the maximum rate of oxygen utilization occurs at the predivision period, with a constant rate through the burst of cytokinesis (there is an apparent decrease by virtue of the doubling in cell number). Figure 56 also shows that cell division can occur at low oxygen tensions and that after it is completed (from the 21st to the 23d hours of the cycle), the rate of respiration is reduced to a level comparable to that of cells in the cold period. This rate is, in fact, considerably lower than that found for exponentially dividing cells at 28.5° (i.e., QO_2 of 60).[10]

These results indicate that in synchrony, induced by a repetitive temperature cycle, there are periods of maximum and minimum respiratory activity, reflecting or possibly contributing to the synchronization process. They also emphasize the need to examine further the energetics of cell division.

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[7] T. W. James, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 104–5.

[8] G. M. Padilla and T. W. James, *Exptl. Cell Res.* **20**, 401 (1960).

[9] J. J. Blum and G. M. Padilla, *Exptl. Cell Res.* **28**, 512 (1962).

[10] B. W. Wilson and T. W. James, *Exptl. Cell Res.*, in press.

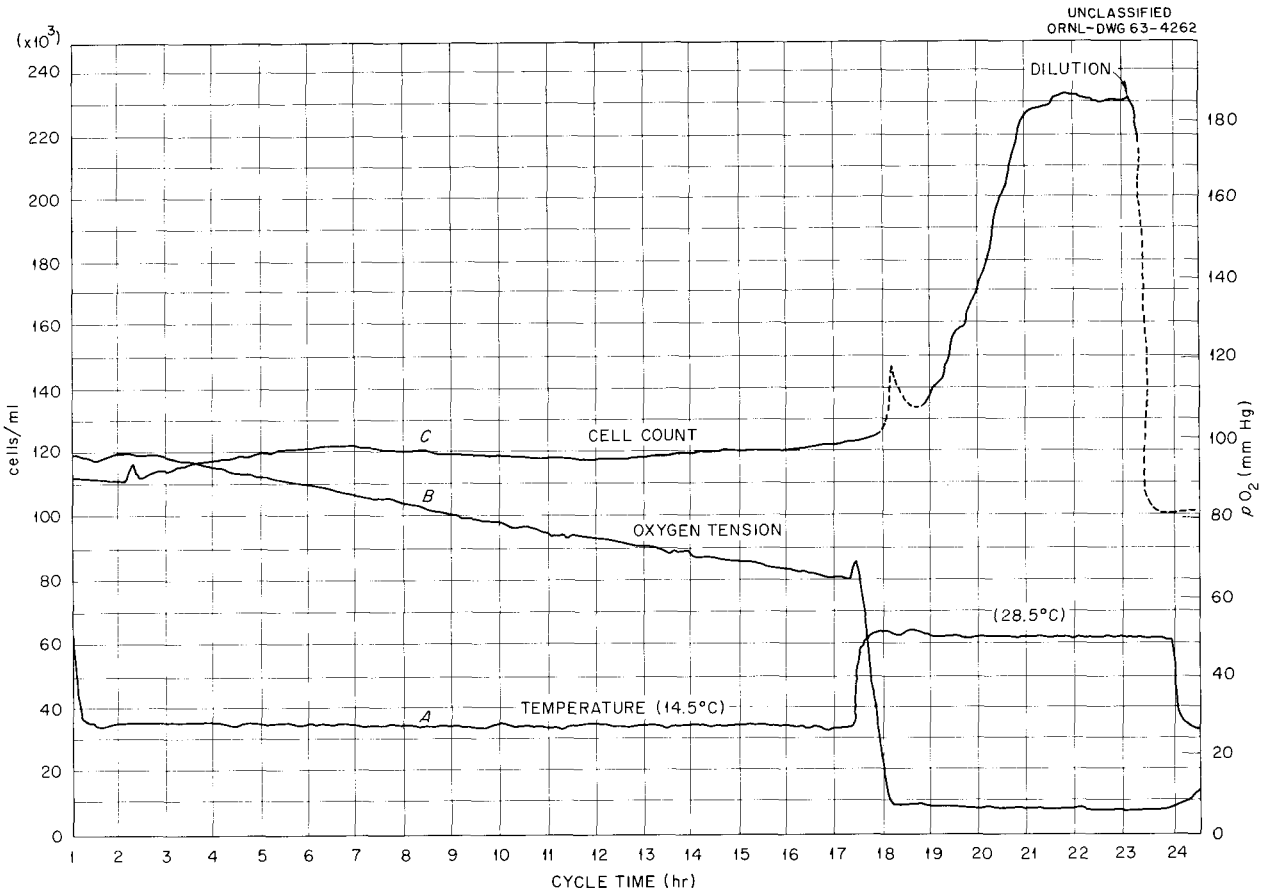

Fig. 55. Continuous Recordings of a Synchronized Burst of Division of the Flagellate *Astasia longa*. Three tracings are shown: (A) temperature record showing the 24-hr temperature cycle (17.5 hr at 14.5°C and 6.5 hr at 28.5°C); (B) dissolved oxygen content of the medium measured with an oxygen electrode and expressed on the right ordinate in mm Hg for the partial pressure of O_2; and (C) cell density recording as measured by a continuous-flow Coulter counter and given on the left ordinate in cells/ml.

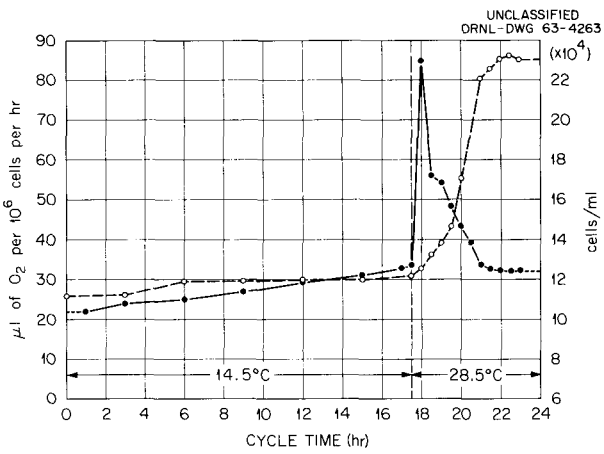

Fig. 56. Respiratory Activity of Synchronously Dividing *Astasia longa*. Changes in QO_2 (expressed on the left ordinate in $\mu l/10^6$ cells/hr) are indicated by the solid line. The changes in population density are given on the right ordinate in cells/ml and are indicated by the broken line.

Chemical Agents and Induction of Synchrony. – The relations existing between the chemical composition of the medium and induction of cell division synchrony are being examined. In particular, the effect of various compounds [such as cysteine, homocystine, mercaptoacetic acid (thioglycolic acid), and ascorbic acid] as well as the trace metals usually found in Cramer-Myers medium[8] is under investigation. It has been found that a reduced sulfur compound is required for enhancement and continued cell division synchrony in *Astasia longa* grown on this chemically defined salt medium (e.g., mercaptoacetic acid at a 5×10^{-5} *M* concentration.)[11] The role of ascorbic acid is as yet unclear, although it appears to lower the respiratory activity of the synchronized cells when it is added at 10^{-3} *M* concentration. The metal components of Cramer-Myers medium[8] can be gradually reduced to one-twentieth their original concentration without adverse effects, if the chelating agent is proportionally reduced. An improvement in the synchrony pattern is evident, although the optimum concentrations of such elements has not yet been determined.

Effects of Low-Level X Radiation. – Although most cells appear to be quite sensitive to x radiation, protozoa generally are very resistant, at least if the criterion of mortality is used as an index of radiation damage.[12] The delay of division as an alternate measure of radiation injury is not easily applicable when exponentially dividing cells are used, particularly if reference is to be made to the disruption of a given process in the cell cycle (e.g., nucleic acid synthesis). Less direct techniques, such as pulse labeling with nucleic acid precursors, must be used.

Synchronously dividing *Astasia longa* were exposed to x radiation with dosages in the range 100 to 400 r, after it had been determined that cell division in exponentially dividing cells can be delayed at these levels of radiation. The cells can withstand exposures of up to 100 kr without loss of viability. Although the effect of x radiation throughout the entire synchronized cell cycle has not yet been examined, it seems that exposure early in the warm period, just prior to mitosis,[9] results in division delays of approximately 0.5 to 1.5 hr for the dose range indicated above. Further investigations are to be done in which concomitant cytological observations and biochemical assays will be performed.

[11] G. M. Padilla and T. W. James, in *Methods in Cell Physiology* (ed. by D. M. Prescott), vol I, Academic Press, New York, in press.

[12] R. F. Kimball, *Ann. Rev. Microbiol.* 11, 199 (1957).

FURTHER STUDIES ON AGGREGATION REACTIONS IN RAT LIVER HOMOGENATES

Herbert Schuel Regina Schuel

When the surface of a cell is ruptured, the emerging cytoplasm usually is not dispersed into the medium, since a new membrane is quickly formed and the flow is thus halted. This process is known as the "surface precipitation reaction" or more briefly as the "SPR," and the molecular mechanisms involved are believed to be analogous to those responsible for the coagulation of mammalian blood.[13] It is possible to study the chemical events taking place *in vitro* by adding calcium ions to tissue breis that had been decalcified prior to homogenization.[14–16] In this way the rupture of the cells is separated in time from the influx of calcium ions.

A previous report described the aggregation reactions induced by calcium and magnesium in rat liver brei low-speed supernatants which contained the microsomes and ribosomes in addition to the soluble phase material.[17] The present report describes similar experiments conducted on the soluble phase fraction of rat liver homogenates.

Experimental. – Adult male Sprague-Dawley rats used in this study were stunned by a blow on the head, decapitated, allowed to bleed for several seconds while suspended by the tail; and the livers were then perfused until completely blanched by a mixture of calcium-free Locke's solution and isotonic (0.109 *M*) sodium citrate. Twenty-five percent homogenates in ice-cold isotonic

[13] L. V. Heilbrunn, *Dynamics of Living Protoplasm*, Academic Press, New York, 1956.

[14] T. Hultin, *Exptl. Cell Res.* 1, 272 (1950).

[15] P. R. Gross, *Trans. N. Y. Acad. Sci.* **20**, 154 (1957).

[16] N. G. Anderson, T. Makinodan, and C. B. Norris, *Exptl. Cell Res.* **22**, 526 (1961).

[17] H. Schuel and R. Schuel, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 117–18.

(1.150 *M*) KCl were prepared in a manually operated glass homogenizer. The brei was then centrifuged for 60 min at 144,880 × g_{max} (40,000 rpm) in the No. 40 rotor of the Spinco model L preparative ultracentrifuge. The fat cap was removed by aspiration, and the supernatant obtained contained the soluble phase material.

Aggregation reactions were studied turbidimetrically at 650 mμ in aliquots of these preparations following the addition of $CaCl_2$ or $MgCl_2$ (final conc, 0.025 *M*). As a control, KCl (final conc, 0.075 *M*) was added to produce the same ionic strength. Previous work has demonstrated that the turbidity changes observed are proportional to the degree of aggregation.[16]

A comparison of the effects of calcium and magnesium on aggregation reaction in rat liver brei over the temperature range 30 to 37°C can be seen in Fig. 57. The data are expressed in terms of the change in optical density at 650 mμ produced during the first 10 min of incubation. Over the range 30 to 34°C, the aggregation induced by calcium is faster than that induced by magnesium. At about 35°C, the aggregation rate of the magnesium-activated preparation began to catch up with the calcium-activated preparation and actually passed it at 36 and 37°C. Soluble phase preparations treated with KCl showed no indication of aggregation when incubated at the test temperatures for 10 min.

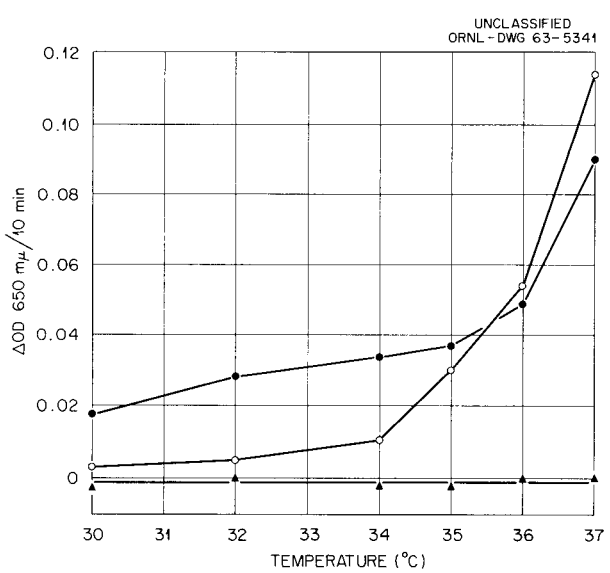

Fig. 57. Effect of $CaCl_2$, $MgCl_2$, and KCl on the Turbidity of the Soluble Phase Fraction of Rat Liver Brei. Data expressed as Δ optical density at 650 mμ for the first 10 min of incubation.

These effects were confirmed in experiments conducted at 30, 35, and 37°C over an incubation period of 45 min. At 30°C, the calcium-induced aggregation was more rapid than the magnesium. At 35 and 37°C, the calcium-activated system aggregated more rapidly at the beginning but was overtaken and passed by the magnesium-treated preparations within 10 to 15 min. Control preparations treated with KCl exhibited a slow, progressive increase in turbidity during the incubation period, but the turbidity changes were much smaller than those in the calcium- and magnesium-activated preparations.

Discussion. – It is known that calcium ions are far more effective than magnesium ions in triggering the SPR in intact cells.[13] Results from previous work with low-speed supernatants (15,000 × g_{max}/0.5 hr) containing the ribosomes and microsomes in addition to the soluble phase material obtained from rat liver homogenates were consistent with these observations, since calcium ions were shown to be more effective than magnesium ions in inducing aggregating reactions.[17] The results reported in this report appear to be quantitatively and qualitatively different. First, the magnitude of the cation-induced aggregation reactions was considerably lower in the soluble phase preparations. Second, after an initial lag the aggregation was greater in the magnesium- than the calcium-treated soluble phase preparations. Additional work is in progress to establish more clearly the relation of these *in vitro* observations to the SPR in intact cells and to isolate and characterize the components of the system.

NATURAL WATER FRACTIONATION PROJECT

W. T. Lammers

Over the past six years, systems have been developed for rapid, quantitative analysis of the particulate materials of natural water. The scope of the investigation has been enlarged and refined as better insights into the nature of the problem developed. It is hoped that the result

of the project will yield better methods for biophysical analysis of natural water and, as a result, a better understanding of biophysical characteristics of cycling in the aquatic environment.

Investigations thus far have concerned the productivity of benthic plants and periphyton in several rivers, and the distribution of radionuclides among the various organic and inorganic particles suspended in the Clinch River. During the study period, basic methods and instrumentation have been developed which can be used to fractionate a natural water sample into concentrated fractions of (1) noncolloidal organic material, (2) noncolloidal inorganic material, (3) colloids and solutes, and (4) pure water. These fractions may be isolated in sufficient quantity for analysis for low-level radionuclide accumulation. This method is useful for routine investigations of biological cycling of such materials as radionuclides and pesticides and for monitoring the physical makeup of a water mass.

Summary of Present Methods. – Natural water samples are first separated into a noncollodial particulate fraction (of mixed organic and inorganic material) and into a colloid-solute fraction by a continuous-flow centrifugation (R_{max}: 7 cm; 28,000 × g; 150 ml/min). This centrifugation also concentrates the noncolloidal fraction of a 26-liter sample into a 300-ml volume. The noncolloidal material is then resuspended and separated into organic and inorganic fractions by density gradient centrifugation (R_{min}: 15 cm; R_{max}: 23.5 cm; 1040 × g, 60 min). As a result of this density gradient centrifugation, approximately 98% of the noncollodial inorganic particles are isolated in the most dense portion of the density gradient, and approximately 99% of the organic particles are isolated in the least dense portion of the density gradient. The separated organic and inorganic fractions may then be removed and prepared for radionuclide or other suitable analyses. Quantitation of the organic material is presently done by wet-weight determinations, while quantitation of the inorganic material is done by dry-weight determinations.

The colloid-solute fraction from the initial continuous-flow centrifugation contains the most surface-active materials of the original sample and has a high content of Ru^{106} and Sr^{90} and other contaminants. Aliquots of this fraction are routinely prepared for analysis by vacuum evaporation at 35°C to prevent loss of volatile materials.

Proposed Investigations. – A continuous-flow density gradient centrifuge rotor would be an ideal means of rapid, one-step separation and concentration of the contents of natural water samples into three fractions: (1) noncolloidal organic particles, (2) noncolloidal inorganic particles, and (3) colloids and solutes. The reduction of lapsed time from sampling until final analysis and the elimination of two steps would increase both the reliability of the analysis and the speed of handling the large volumes of material and large numbers of samples required. Several models of such a rotor have been designed, fabricated, and tested. The models tested so far have shown that the concept is feasible. A new modification of the rotor has been fabricated and is in the testing stage. When this rotor is perfected, it should be possible to isolate groups of organic particles of the same density into isodensity zones and thus be able to measure not only the total amount of organic material but also the amount of several different types of organic material which may be present.

At present the colloids and solutes from the initial continuous-flow centrifugation of the natural water sample are analyzed as a single fraction. This is not a homogenous fraction; it is composed of virus particles, protein molecules, colloidal clay, and dissolved or ionized inorganic material. In several initial experiments, colloids (molecular weight greater than ~6000) have been separated from solutes (molecular weight less than ~6000), and the results indicate that the highly surface active colloids adsorb most of the radionuclide activity found in the colloid-solute fraction. In another series of investigations the virus particles of a natural water sample have been bulk isolated (5×10^{10}/ml) from the other organic and inorganic materials and identified by uv absorption at 260 and 280 mμ. The isolation and detection of large numbers of virus particles from natural water is more difficult than that of bacteria, but at present viruses are coming under increasing scrutiny. In addition, little is known about the role virus particles in water may play in cycling radionuclides and other contaminants. It is therefore of interest to follow the cycle and course of virus particles in natural water that may ultimately be used for human consumption.

While the noncolloidal organic fraction of the water sample is at present quantitated by wet-weight determinations, better means of estimating

the meaning of a given amount of organic material are needed. The plankton content of streams below dams is usually so low that bulk isolation procedures must be used to obtain enough material for analysis, and direct measurement of productivity is not feasible. At present one-fifth of the normal (control) metabolic activity remains in plankton removed from an umbradil-salt density gradient. Since no metabolic activity was found in plankton removed from sucrose or 2-bromoethanol density gradients, the use of umbradil-salt density gradients has materially improved the estimates of productivity of the original samples. Investigations are under way to increase the amount of metabolic activity remaining after density gradient centrifugation. Eventually, not only bulk organic material should be measured for productivity but also the various groups of organic particles such as diatoms, bacteria, and green algae should be isolated and subjected to productivity studies. If a high level of metabolic activity can be obtained in isolated fractions, then a ratio of productivity/weight, or number, of particles could give a number which would express both metabolic ability and total number of particles and thus give a good measure of what may be expected in terms of accumulation, carbon fixation, and cycling.

CARBOHYDRATE ANALYZER: DESIGN IMPROVEMENTS AND EXPERIMENTAL APPLICATION

J. G. Green N. G. Anderson

Introduction. – The progress in the design of a workable automatic carbohydrate analyzer has been reported previously,[18,19] and the present status of the machine is described in detail elsewhere.[20] However, some previously unreported design changes and analytical applications are reported here.

Experimental. – The use of very fine resins, preferably in the 5- to 15-μ size range, materially improves the resolving capability of a column but substantially increases the pressure required to obtain satisfactory elution flow rates. Initially, jacketed glass columns employing ball-and-socket joining and sintered glass plates were used; however, these columns were not suitable for elution pressures in excess of 80 psi. To evaluate the performance of very fine resins at elevated column pressures, columns have been fabricated using "Swagelok" fittings (Crawford Fitting Company, Cleveland, Ohio) fitted to jacketed, heavy-walled borosilicate glass tubing. Columns have been made from $\frac{1}{2}$-in.-OD by $\frac{5}{16}$-in.-ID tubing using fitting 810-6-4-316 and from $\frac{3}{8}$-in.-OD by $\frac{3}{16}$-in.-ID tubing using fitting 600-6-4-316. As recommended by the manufacturer, Teflon front and nylon back ferrules are employed. In addition, to cushion and seal the tubing within the fitting, a $\frac{1}{32}$-in. Teflon gasket is fitted between the end of the tubing and the inside seat of the fitting body. The fitting used at the base of the column is counterbored to accept a 5-μ-porosity sintered steel disk supplied in the "Swagelok" snubber fitting. To minimize dead space, an adapter machined from polyvinyl chloride and fitted with capillary Teflon tubing is press-fitted against the sintered plate. These columns have been routinely used at a pressure of 160 psi and tested to 200 psi, with no evidence of failure; however, the fittings must be periodically retightened, since the Teflon front ferrule tends to flow slightly under compression.

Although Dowex 1-X8 resin is routinely used in the analyzer, resins with lower cross linkage have been assayed and found to yield somewhat better component separations; however, the stability of such resins under elevated pressures has not been assayed in the analytical system. A 10% cross-linked resin yields essentially the same resolution as the 8% form.

The output stream from the colorimetric processing system is now being monitored by a Technicon colorimeter fitted with the nonvented flow cell and ratio recording system, which yields a readout superior to the system described in previous reports. Air bubbles are removed from the processed stream at a vented trap, and the entire stream is monitored without requiring a proportioning pump. The rate of flow through the cell is established by the difference in height between the inlet and outlet streams of the cell.

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[18] J. G. Green and N. G. Anderson, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, pp 128–30.

[19] J. G. Green and N. G. Anderson, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 118–20.

[20] J. G. Green and N. G. Anderson, *Joint NIH–AEC Zonal Centrifuge Development Program Semiann. Rept. January 1–June 30, 1963*, ORNL-3502 (Special).

The analyzer has been applied to the sorbose toxicity problem in Neurospora discussed by de Serres *et al.*[21] and by Brockman and de Serres.[22] The hydrolysis of sucrose during sterilization of the sorbose-sucrose medium has been confirmed and will be evaluated further by means of the automated system.

Discussion. – The feasibility of the automated system for carbohydrate analysis has been established. Current efforts are being expended to produce a fully practical, trouble-free version of the analyzer suitable for everyday laboratory use. A more durable pump for the dispensing of the concentrated sulfuric acid and phenol reagents is being sought, and attempts are being made to replace fragile glass components with corrosion-resistant metal and plastic counterparts. The use of refractometric monitoring to monitor preparative column outputs is being studied, although such monitoring has been found to be too insensitive for analyses in the micromolar range assayed colorimetrically in the present system. In addition, the investigation of sample preparation techniques is under way.

FLOW OF WATER IN MULTICOMPARTMENTED CELLS AND TISSUES DURING FREEZING

Peter Mazur

Introduction. – Arguments have been presented elsewhere that the presence or absence of large ice crystals within a cell is one of the important factors determining injury.[23,24] One argument is based on calculations of the water content of cells at different subzero temperatures as a function of cooling rate, permeability to water, and a number of other parameters.[25] These physicochemical calculations were restricted to single, isolated cells suspended in an aqueous medium and they estimated only the water content of the cell as a whole.

Actually, cells are not single, homogeneous compartments of aqueous solution separated from the external medium by a single barrier, the cell membrane. Rather, they are multicompartmented systems in which various packets of solution (e.g., nucleus, mitochondria) are, in turn, separated from other portions of the cytoplasm by membranes. For this reason and also because cells are often aggregated into tissues, it seemed desirable to investigate the theoretical changes in water content in multicompartmented systems at subzero temperatures.

There was another reason to calculate the water content and hence the likelihood of intracellular freezing in tissue cells. Salt[26] and Sherman[27] recently claimed that the cells of tissues can contain ice and still be viable; therefore, they concluded that the location of ice crystals is not an important factor in cell injury. For example, Sherman found that sections of mouse skin remain viable when frozen slowly at 1°/min but not when frozen rapidly, the criterion of viability being chiefly the ability of the thawed tissue to "take" when grafted on a recipient mouse. This protective effect of slow cooling agrees with that found in many single cells. In single cells, slow cooling is believed to protect because it produces dehydration of the cells and so precludes intracellular freezing. But in skin, Sherman found that *both* slowly and rapidly cooled cells contain ice; moreover, he found that the ice crystals in slowly cooled cells are considerably larger than those in rapidly cooled cells, in spite of the fact that only the former yielded viable grafts. This conclusion is contrary to that reached for single cells, where the evidence from a number of investigators (including Sherman) indicates that the presence of large intracellular ice crystals is probably always lethal.

Methods. – The starting point for the calculations will be a cell or tissue surrounded by ice and cooled to the freezing point of the protoplasm (~0.6°C). As cooling progresses further, we shall assume that the protoplasm does not freeze, but becomes supercooled. A supercooled liquid (by definition) has a higher vapor pressure than ice, and as the temperature is lowered, the difference between the vapor pressure of ice and

[21] F. J. de Serres, H. G. Kolmark, and H. A. Brockman, *Nature* **193**, 556 (1962).

[22] H. E. Brockman and F. J. de Serres, *Am. J. Botany*, in press.

[23] P. Mazur, *Ann. N.Y. Acad. Sci.* **85**, 610 (1960).

[24] P. Mazur, *Biophys. J.* **3**, 323 (1963).

[25] P. Mazur, *J. Gen. Physiol.*, in press; also see preceding semiannual reports.

[26] R. W. Salt, *Can. J. Zool.* **39**, 349 (1961).

[27] J. K. Sherman, *Anat. Record* **144**, 171 (1962).

that of an ideal supercooled solution in a singly compartmented cell has been shown[25] to be

$$\frac{d \ln p_e/p_i}{dT} = \frac{L_f}{RT^2} - \frac{nv}{(V + n_2 v)V}\frac{dV}{dT}, \qquad (1)$$

where

p_e, p_i = external and internal vapor pressures,

T = temperature,

L_f = latent heat of fusion,

R = gas constant,

n = osmoles of intracellular solute,

v = molar volume of water,

V = volume of intracellular water.

Since $p_i > p_e$, water will flow out of the cell; and if the membrane is permeable only to water, the V rate of change will be

$$\frac{dV}{dt} = \frac{kART}{v} \ln p_e/p_i , \qquad (2)$$

where t is time, k is the permeability constant for water, and A is the area of the cell membrane.

In general, k is an exponential function of temperature, so that

$$k = k_g e^b(T - T_g) , \qquad (3)$$

where k_g is the known constant of temperature (T_g) and b is the temperature coefficient of k.

If the cooling rate is held constant so that

$$\frac{dT}{dt} = B , \qquad (4)$$

Eqs. (1) to (3) can be combined to give

$$Te^{b(T_g - T)}\frac{d^2V}{dT^2} - \left[(bT + 1)e^{b(T_g - T)}\right. - \frac{ARk_g n}{B(V + n_2 v)}\frac{T^2}{V}\frac{dV}{dT} = \frac{L_f A k_g}{Bv}. \qquad (5)$$

The full derivation of this equation and a discussion of the underlying assumptions are given elsewhere.[25] Equation (5) thus describes the water content of *singly* compartmented cells as a function of temperature and several parameters.

Figure 58 depicts a simple geometry for a *multicompartmented* system, namely, a three-layered sphere. Comparable Eqs. to (1) to (3) for this system are as follows:

The vapor pressures of water in the external ice and in the three layers will be

$$\frac{d \ln p_e}{dT} = \frac{L_s}{RT^2} , \qquad (6)$$

$$\frac{d \ln p_\alpha}{dT} = \frac{L_v}{RT^2} + \frac{n_\alpha v}{(V_\alpha + n_\alpha v)V_\alpha}\frac{dV_\alpha}{dT}, \qquad (7)$$

$$\frac{d \ln p_\beta}{dT} = \frac{L_v}{RT^2} + \frac{n_\beta v}{(V_\beta + n_\beta v)V_\beta}\frac{dV_\beta}{dT}, \qquad (8)$$

$$\frac{d \ln p_\gamma}{dT} = \frac{L_v}{RT^2} + \frac{n_\gamma v}{(V_\gamma + n_\gamma v)V_\gamma}\frac{dV_\gamma}{dT}, \qquad (9)$$

where L_s and L_v are the latent heats of sublimation and vaporization.

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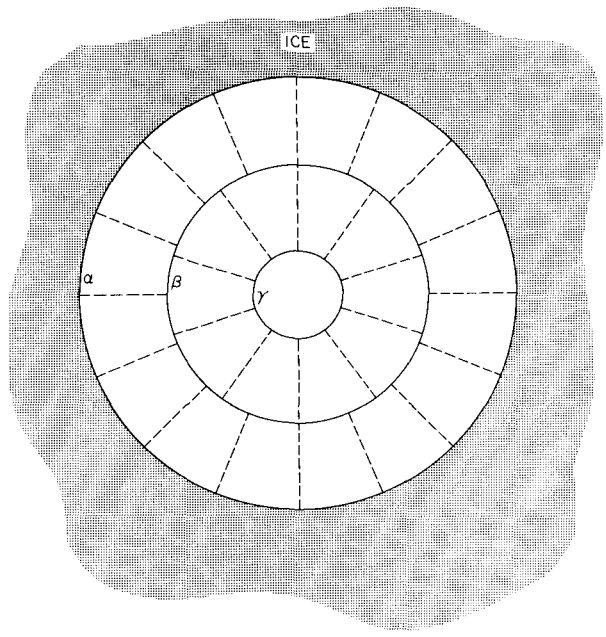

Fig. 58. A Three-Layered Spherical Multicompartmented System Surrounded by Ice. The membranes separating layers α, β, γ from each other and the surrounding ice are assumed to be permeable only to water. Dotted lines represent semipermeable membranes between compartments (or cells) within a layer.

Once again, the rate at which water leaves a layer will be proportional to the difference in vapor pressure between that layer and an adjoining one. The rate of *change* of water in any given layer, however, will equal the rate at which water moves from the layer to one external to it *minus* the rate at which water enters the layer from one internal to it.

Thus in our spherical model

$$\frac{dV_\alpha}{dt} = \frac{k_\alpha A_\alpha RT}{v} \ln p_e/p_i - \frac{k_\beta A_\beta RT}{v} \ln \frac{p_\alpha}{p_\beta}, \quad (10)$$

$$\frac{dV_\beta}{dt} = \frac{k_\beta A_\beta RT}{v} \ln p_\alpha/p_\beta - \frac{k_\gamma A_\gamma RT}{v} \ln \frac{p_\gamma}{p_\beta}, \quad (11)$$

$$\frac{dV_\gamma}{dt} = \frac{k_\gamma A_\gamma RT}{v} \ln \frac{p_\gamma}{p_\beta}. \quad (12)$$

The terms $k_{\alpha\beta\gamma}$ and $A_{\alpha\beta\gamma}$ refer to the membranes between α and ice, β and α, and γ and β respectively. Since all sectors in a given layer are equidistant from the ice which is providing the vapor pressure driving force, and since we are assuming thermal equilibrium throughout, all diffusion will be toward the ice and there will be none between compartments or between cells in a given layer.

Again, assume the cooling rate constant and identical in all regions so that $dT/dt = B$ and assume that k_α, k_β, and k_γ follow Eq. (3).

In theory, it would be possible to combine Eqs. (6) to (12) and (4) and the three equations for k_α, k_β, and k_γ analogous to Eq. (3) to obtain three equations comparable to Eq. (5); i.e., an equation for each layer giving its water content (V) as a function of T, of the water contents of the other layers, and of other parameters applicable to all three layers. In programming for actual numerical solutions, however, Harkrider[28] finds it better to introduce the individual equations and allow the simulaneous solutions to be carried out by the computer. He is, however, eliminating Eqs. (3) and (4) first by hand substitution.

Results and Discussion. – Further progress in this analysis requires answers to four important questions: (1) What simplifying assumptions can or should be made? (2) Are appropriate values for the parameters available in the literature, and what specific values should be used? (3) What geometries of multicompartmented systems should be considered? (4) Are numerical solutions feasible with a reasonable expenditure of computer time?

As for the first question, the following is being assumed: (1) all parameters except V and T are considered constant in any one solution, (2) k_g and b are assumed equal in all layers, (3) all compartments are considered to be isoosmotic with each other above 0°C and therefore of equal osmolarity.

A search of the literature is yielding to values of the parameters m_i (intracellular osmolality), b, k_g, T_g, and V_i (volume of water in cells at normal temperatures). Values for R, v, L_s, L_v, and L_f are known with high accuracy. The cooling rates (B) of interest will be 1, 10, and 100° and, perhaps, 1000°C/min. The remaining parameters can be derived from the above mentioned.

Initial computations will assume the spherical model of Fig. 58 for layers 1, 3, 6, and 12 μ thick. Calculations will also be made for other geometries. These are: a sphere with the ice in the center; an infinitely long three-layered cylinder with the ice either outside or in the center of the cylinder; an infinitely long and wide three-layered slab with ice on both sides and perhaps on one side. The thicknesses of the layers will probably be restricted to 1, 3, 6, and 12 μ.

To investigate the feasibility of numerical solutions, attempts were made to solve the equations using the specific values for the parameters listed in Table 35. Values for d_i, m_i, b, and k_g are "typical" values for single cells.[25] Solutions requiring about 20 min of computer time were obtainable; that for a cooling rate of 10°C/min is shown in Fig. 59.

At this point, only two aspects of these results require mention:

1. The curve labeled "equilibrium" shows the percentage of the initial water that would remain

[28] M. T. Harkrider, Mathematics Division, ORNL, is carrying out all programming for obtaining numerical solutions by the Runge-Kutta procedure. The computer is a Control Data Corporation machine.

Table 35. Parameters for Solution of Equations for the Kinetics of Water Loss in Multicompartmented Systems

(The configuration is a three-layered sphere surrounded by ice)

Symbol	Values	Description	Units
d_i	5.84	Thickness of each layer	μ
m_i	0.5	Intracellular osmolality	moles/1000 g of H_2O
p_{ei}	5.475×10^{-3}	Vapor pressure of ice at T_f	atm
T_f	272	Freezing point of protoplasm	$^{\circ}K$
n_α	4.323×10^{-15}	Osmoles of solute in α	moles
n_β	1147×10^{-15}	Osmoles of solute in β	moles
n_γ	44.1×10^{-15}	Osmoles of solute in γ	moles
$V_{\alpha i}$	8646	Initial volume of H_2O in α	μ^3
$V_{\beta i}$	2294	Initial volume of H_2O in β	μ^3
$V_{\gamma i}$	88.2	Initial volume of H_2O in γ	μ^3
A_α	2679	α-ice interfacial area	μ^2
A_β	964.3	$\alpha\beta$ interfacial area	μ^2
A_γ	107.1	$\beta\gamma$ interfacial area	μ^2
B	−10	Rate of temperature change	$^{\circ}K$/min
b	0.0325	Temperature coefficient of k_g	deg^{-1}
k_g	0.3	Permeability of H_2O at T_g	$\mu\ min^{-1}\ atm^{-1}$
T_g	293		$^{\circ}K$
R	82.057×10^{12}	Gas constant	μ^3 atm $mole^{-1}$ deg^{-1}
v	18×10^{12}	Molar volume of H_2O	μ^3/mole
L_s	50.37×10^{16}	Latent heat of sublimation	μ^3 atm $mole^{-1}$
L_v	44.44×10^{16}	Latent heat of vaporization	μ^3 atm $mole^{-1}$
L_f	5.93×10^{16}	Latent heat of fusion	μ^3 atm $mole^{-1}$

in each layer if cooling were infinitely slow (i.e., as dT/dt approaches zero). The differential equation of this relation can be solved analytically,[25] the integrated equation being

$$\ln\left[\frac{V/V_i}{(V/V_i) + 0.018\ m_i}\right] = \frac{L_f}{R}\left(\frac{1}{273} - \frac{1}{T}\right). \quad (13)$$

It will be noted that the nonequilibrium curves for regions α, β, and γ when $B = -10^{\circ}$/min eventually merge precisely with the equilibrium curve. This, along with the continuous nature of the curves, attests to the correctness of the numerical solutions.

2. At a temperature of −9°C, the water content of the outer layer (α) is only 12% of its initial value. That water is supercooled only 2° (horizontal distance to equilibrium curve), and is, therefore, unlikely to freeze. But the water content of the innermost layer (γ) at −9°C is still 72% of its original value and is supercooled nearly 8°. It, therefore, is much more likely to freeze. In other words, these very preliminary solutions suggest that in multicompartmented systems, some regions may dehydrate without freezing; other regions may freeze before they have been able to dehydrate.

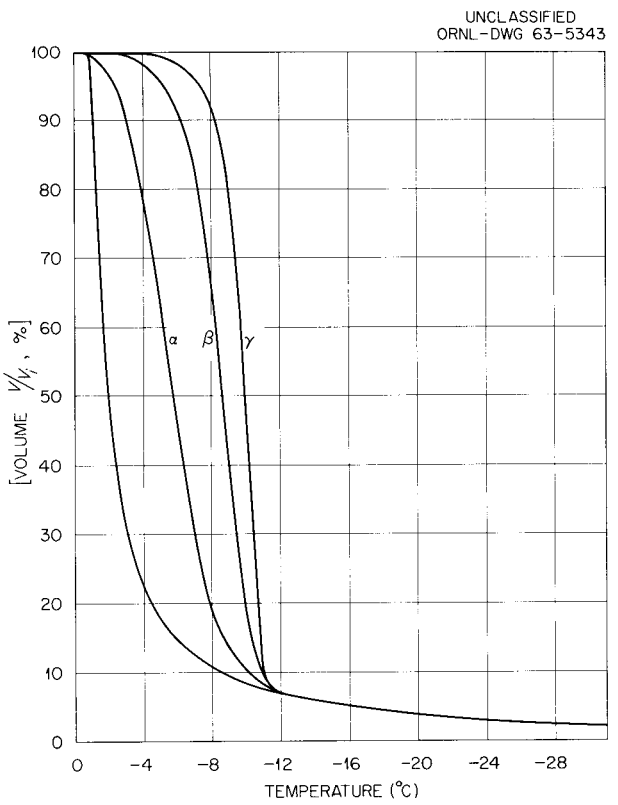

Fig. 59. Numerical Solution Showing the Water Contents in Each Layer of the Model Depicted in Fig. 58 as a Function of Temperature. The parameters used in the solution are given in Table 35.

DENSITY GRADIENT CENTRIFUGATION IN ANGLE-HEAD ROTORS

W. D. Fisher G. B. Cline N. G. Anderson

Introduction. – Preparative isopycnic density gradient centrifugation is ordinarily done in high-speed, swinging-bucket rotors, such as the Spinco SW 39 and SW 25. In addition to requiring this special equipment, the technique is severely limited by the small number of samples and the low total sample volume that may be used. In recent studies on methods for isolating small numbers of virus particles from tissues and for separating nucleic acids which differed in density, we required means for centrifuging many samples simultaneously. The possibility of making such separations in ordinary angle-head rotors, which are widely available and have both a large number of tubes and high capacities, was therefore investigated. This application of angle-head rotors depends on reorientation of the density gradient from horizontal to vertical during deceleration, which, in effect, replaces the mechanical transition of the tubes in the swinging-bucket rotor. This approach appeared feasible since the same basic principle has been applied to the construction of hollow, cylindrical, reorienting gradient rotors (C series rotors) in which a density gradient formed at rest is reoriented radially during centrifugation and is subsequently recovered at rest after deceleration.

Results and Discussion. – Figure 60 illustrates the principal results obtained in a test system of T3 bacteriophage in CsCl (ρ = 1.519). After the rotor had coasted to rest without braking, the tubes were removed and unloaded through a length of stainless steel tubing inserted into the bottom of the tube. The sample was passed through a flow cell in a Beckman DB spectrophotometer and a Waters model 34H refractometer which displayed optical density at 260 mμ and refractive index on a Brown 12-point recorder. The results show (1) that the gradient produced approached the predicted equilibrium condition, (2) that good banding was obtained, and (3) that the reorientation of the gradient column did not cause appreciable disturbances of the gradient or sample zone.

Results similar to those for the No. 40 rotor were obtained with the Nos. 30 and 50 Spinco rotors. DNA, adenovirus, and R17 bacteriophage have been separated isopycnically in angle-head rotors.

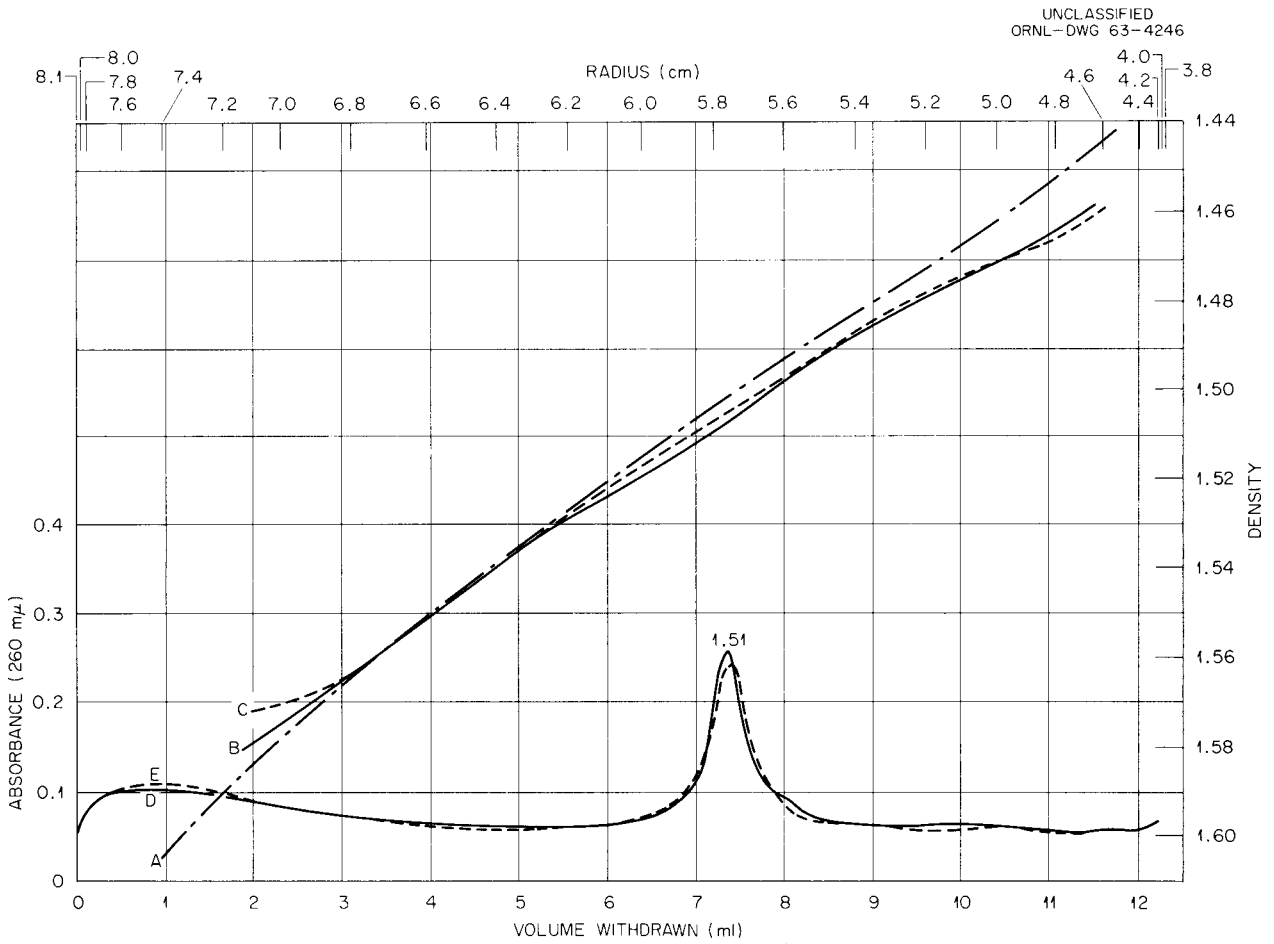

Fig. 60. T3 Bacteriophage Banded in CsCl. Curve A is the calculated gradient at equilibrium. Curve B was obtained after centrifuging for 30 hr at 33,000 rpm in a No. 40 rotor in a Spinco model L centrifuge. Curve D is the corresponding optical density tracing. Curves C and E were obtained after a 1-min recentrifugation at 33,000 rpm of previously banded phage.

Nucleic Acid Chemistry

Nucleotide Chemistry

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Molecular Biology

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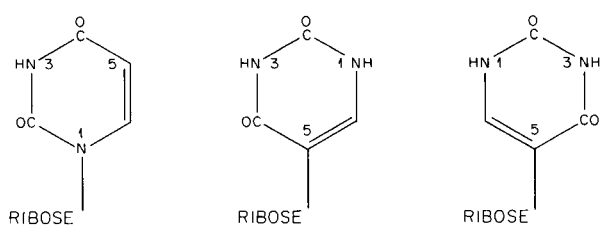
POLYPSEUDOURIDYLIC ACID: SOME PHYSICAL AND BIOCHEMICAL PROPERTIES[1]

Waldo E Cohn

Introduction. – Pseudouridine may be regarded as a "natural analog" of uridine. The location of the ribosyl moiety (on carbon 5 instead of the usual nitrogen 1) rotates the uracil ring 120° from its position in uridine. Viewed perpendicularly to its plane we thus find the H-bonding positions of uridine (N3-C4) replaced by either N3-C2 or N1-C2 groups, the former being favored by the formation of cyclo-pseudouridine:



URIDINE PSEUDOURIDINE

Therefore, pseudouridine, in a polymer, should be able to form the same H-bonds as does uridine, although with a different CO–HN group. From the standpoint of nearest-neighbor interactions, there is again great similarity; in the cyclo-pseudouridine conformation, there are four out of six atoms in the ring in nominally equal positions.

From this, it may be argued that pseudouridine should be generally similar to, yet somewhat different from, uridine in its behavior in biochemical systems. From published work, this appears to be the case. ΨDPG can substitute for UDPG in glucose transfer, but not as efficiently. ΨTP can substitute for UTP in a polymerization reaction (requiring all four triphosphates) but fails of equality when both are present and presents a slightly altered set of "nearest-neighbor" relations in the resulting polymer.

Hence neither theory nor practice predicts definitely the behavior of polypseudouridylic acid (poly Ψ) nor of mixed polymers of Ψ and U [poly (Ψ,U)]. Nor has the polymerization of ΨDP, alone or with another diphosphate, been effected.

The present research constitutes a preliminary exploration of the route to such polymers with polynucleotide phosphorylase and some properties of the polymers formed.

[1]This work was done in collaboration with M. Grunberg-Manago, A. M. Michelson, F. Pochon, and L. J. Dondon while W. E Cohn was on leave at the Institut de Biologie Physico-Chimique, Paris.

Pseudouridine, as the 2′,3′-*O*-isopropylidene derivative, was phosphorylated by the cyanoethylphosphate method to 5′-ΨMP, which was converted to ΨDP with diphenylphosphochloridate. The ΨDP was polymerized, either alone or with added UDP, by enzymes from *Azotobacter vinelandii* and *Escherichia coli* to yield three polymers: poly Ψ, poly (Ψ_1, $U_{2.4}$), and poly (Ψ_1, U_{20}).

Results. – The poly Ψ had the unexpected high S_{20} value of 9 and displayed a definite "melting" point at about 60°C, which became somewhat lower as the pH approached the pK (9.6) of pseudouridine. Poly (Ψ_1, $U_{2.4}$) showed no such behavior, nor does poly U. Poly Ψ also forms a three-membered complex with poly A in the proportion of 2Ψ to 1A.

In an amino acid incorporation system, the poly Ψ described above was totally inactive toward phenylalanine, whereas the poly (Ψ_1, $U_{2.4}$) was as active, per mole of P, as an equivalent amount of poly U. The latter polymer was quite active, in one trial, toward leucine as well, but it is not known if the degree of activity here is beyond what might be found in poly U and, therefore, in the poly (BrU) range.

Discussion. – The evidence for secondary structure in poly Ψ (S_{20} value of 9 and a typical "melting" curve) is somewhat surprising, for it contrasts markedly with poly U. It seems reasonable to attribute this behavior to the additional NH group in Ψ, possibly acting through the C2 group that now finds itself between two NH groups. This offers additional H-bond possibilities, both inter- and intra-chain. The possibility that the latter is the more important is supported by the absence of structure in the copolymer poly (Ψ_1, $U_{2.4}$) where U occupies only 30% of the positions in a chain of poly U, for it can be argued that *inter*-chain binding should be less diminished by such dilution with U than would the *intra*-chain binding.

Regardless of mechanism or form, the inactivity of poly Ψ in promoting phenylalanine incorporation may properly be ascribed to the presence of secondary structure, which has been shown to inhibit the activities of polynucleotides in such an incorporation system. The possibility that Ψ is inactive or inhibiting in itself is eliminated by the complete activity of the (Ψ_1, $U_{2.4}$).

PURIFICATION OF DEOXYNUCLEOTIDE POLYMERIZING ENZYMES

Masahiko Yoneda
Patricia A. Williams
G. E. Houts
F. J. Bollum

A pilot-scale purification process (called "P-70 process") for the deoxynucleotide polymerizing enzymes of calf thymus gland was described briefly in the last progress report,[2] and it now provides adequate material for further study and purification. We are engaged, therefore, in a detailed study of the fractions obtained by chromatography of P-70 process material on hydroxylapatite and Sephadex G-100.

Gradient elution chromatography on hydroxylapatite at pH 7.0–7.2 (potassium phosphate) separated DNA polymerase from all DNase activity[3] but at this pH "end-addition" enzyme (ref 4) eluted with the DNA polymerase. Since it was known that the polymerase and end-addition enzymes are separate proteins,[5] a detailed examination of the effect of pH on the DNA polymerase–end-addition enzyme separation was carried out. At pH 7.5 a good separation is achieved (Fig. 61; fraction I is end-addition enzyme and fraction II is DNA polymerase), and both proteins appear to be free of phosphodiesterase and DNase activity.

Separation of the two deoxynucleotide polymerizing enzymes may also be effected by chromatography on Sephadex G-100 at pH 7.2 in 0.05 *M* phosphate buffer. As shown in Fig. 62, DNA-polymerase and end-addition enzyme were eluted at 1.1 and 1.7 holding volume, respectively, on G-100. These results show that the molecular weight of DNA-polymerase is greater than 100,000, while that of the end-addition enzyme is less than 100,000.

These separations are important new steps forward in this program. The products formed by both of the deoxynucleotide polymerizing enzymes

[2]M. Yoneda and F. J. Bollum, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 121.

[3]F. J. Bollum, B. Alberts, and P. A. Williams, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1962*, ORNL-3267, pp 112–13.

[4]F. J. Bollum, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1960*, ORNL-2193, pp 108–9.

[5]Eleanor Groeniger and F. J. Bollum, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p 139.

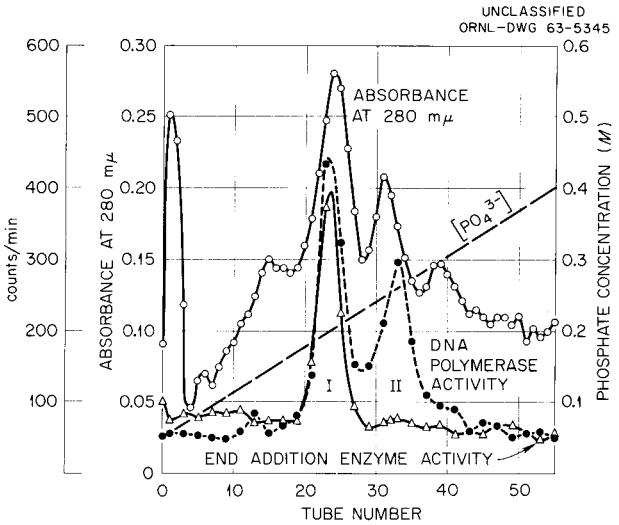


Fig. 61. Chromatography of P-70 Process Fraction on Hydroxylapatite at pH 7.5.

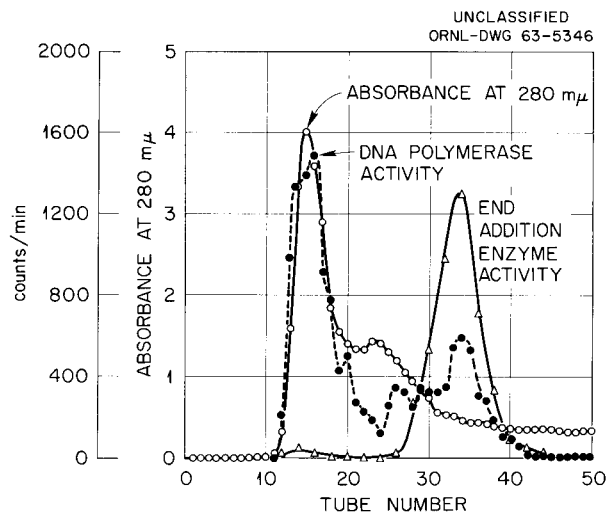


Fig. 62. Gel Filtration of P-70 Process Fraction on Sephadex G-100 at pH 7.2.

are of considerable interest and are discussed separately in this report.

ENZYMATIC SYNTHESIS OF DEFINED POLYDEOXYNUCLEOTIDES

Eleanor Groeniger F. J. Bollum

A study of the "end-addition" activity in calf thymus DNA polymerase utilizing oligodeoxyribonucleotide primers revealed that this enzyme was different and separable from DNA polymerase.[5] Since then, separation of the two enzymes in good yield has been achieved with hydroxylapatite and Sephadex G-100 columns.

With this "end-addition" enzyme (fraction I from hydroxylapatite), it is possible to synthesize polymers of the type $(pT_3)(pX)_n$ using $d(pT)_3$ as primer. The molecular size, spectra, hyperchromicity, and chromatographic properties of some of these polymers have been studied. In particular, a polymer designated $d(pT)_3(pA)_{33}$ has been analyzed for base composition and hypochromicity. Base analysis shows the presence of only adenine and thymine in the designated quantities. Degradation by venom phosphodiesterase produces a 50% hyperchromicity, suggesting some secondary structure in this polymer. Elution from DEAE by 0.6 *M* NaCl confirms the high molecular weight of the product.

Future work will involve use of each of the other bases to form polymers, studies on the physical properties of these polymers, and studies on their ability to serve as primers for complementary synthesis by DNA polymerase (fraction II from hydroxylapatite).

ENZYMATIC SYNTHESIS OF DNA

F. J. Bollum A. Jeannine Bandy

A study of the products of calf thymus DNA polymerase (containing the polymerase and contaminating end-addition enzyme) demonstrated that separation of the two strands did not occur.[6] We have now prepared similar hybrid products (*Escherichia coli* DNA primer labeled with H^3 and bromouracil or bromocytosine triphosphate labeled with P^{32}) using fraction II from hydroxylapatite which contains no end-addition activity.

Density gradient analysis of heat denatured fraction II products again show that strand separa- does not occur, and the earlier result was therefore not the result of contaminating end-addition enzymes.

[6]F. J. Bollum, G. E. Houts, and A. Jeannine Bandy, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 121–22.

Nucleic Acid Enzymology

Elliot Volkin	
Microbial Systems	**Developmental Biochemistry**
Elliot Volkin J. X. Khym J. L. Fairley[a] M. Helen Jones	F. J. Finamore C. G. Mead N. G. Sansing[b] Rose P. Feldman
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BIOCHEMICAL TRANSFORMATIONS OF BACTERIOPHAGE T2 DNA

E. Volkin M. Helen Jones

Introduction. – It has been shown by Stent *et al.*[1] and Kozinski and Kozinski[2] that DNA of infecting T4 undergoes fragmentation before becoming incorporated into progeny molecules. In those progeny DNA molecules containing parental fragments, the average contribution of parental DNA is about 5 to 15% of the progeny DNA. The host cell apparently can bring about breakage and rejoining of the DNA chain. It is noteworthy that the phage has a high rate of genetic recombination.

The experiments reported here are designed to investigate the specificity of the breakage and rejoining phenomenon at the nucleotide level. Bacteriophage T2r+, whose biochemical and genetical properties are similar to T4, is used throughout.

Results. – By enzymically hydrolyzing progeny and parental DNA to the constituent 5′ nucleotides, it was observed that a significant reduction in the contribution of parental hydroxymethyldeoxycytidylic acid (HMCP), relative to the other nucleotides, occurs during the parental DNA to progeny DNA transfer. Thus, if infection is carried out with T2 parent containing DNA uniformly P^{32}-labeled, progeny is obtained whose HMCP contains only about 80% of the P^{32} content relative to the other nucleotides. This result is obtained whether the hydrolysis procedure is carried out with (1) heated DNA and *Escherichia coli* diesterase or (2) a combination of pancreatic DNase and snake venom diesterase.

Essentially the same result exists under conditions of low or high multiplicities of infection with labeled phage parent. Where *de novo* DNA synthesis is largely prevented by the addition of 5-fluorodeoxyuridine, the small yield of progeny phage obtained exhibits a similar reduction in its content of parental $HMCP^{32}$. No differences in results were detected in the DNA's of early or late progeny phage after infection with uniformly labeled T2 parent.

It is known that early progeny phage are enriched in parental DNA material, relative to those synthesized late. Thus it was surmised that if the converse experiment was carried out, whereby unlabeled phage was used to infect P^{32}-labeled

[1]G. S. Stent, G. H. Sato, and N. K. Jerne, *J. Mol. Biol.* 1, 134 (1959).

[2]A. W. Kozinski and P. B. Kozinski, *Virology* **20**, 213 (1963).

bacteria, the relative contribution of P^{32} to HMCP of early progeny phages should be exaggerated. For this purpose, unlabeled T2 was allowed to infect *E. coli* B under conditions where the phosphorus of the host and medium was maintained at constant specific radioactivity at all times. Thus, any perturbations in relative contributions to the newly made phage DNA would not be due to changes in specific activity of the nucleotide precursors. It was observed that the labeling in progeny HMCP was notably elevated in comparison with the other three deoxynucleotides.

Experiments were designed to determine whether the relative reduction in transfer of HMCP involved the entire nucleotide or just the nucleotide phosphorus. Parental T2 was used containing not only P^{32} in the DNA but tritium label in the DNA pyrimidines. It was observed that, along with the relative reduction in parental contribution to progeny $HMCP^{32}$, there was a similar reduction in HMC tritium. Thus, it would appear that the phenomenon is associated with the entire HMCP nucleotide rather than the HMC phosphorus moiety alone.

Discussion and Summary. – These data may be interpreted in terms of a tentative working hypothesis. Parental T2 DNA undergoes fragmentation at specific sites that are relatively enriched in HMC nucleotides. A partial hydrolysis of nucleotides along the chain occurs which must be repaired for polynucleotide chain rejoining to occur. These "labile" sites are the loci involved in genetic recombination.

The possibility exists that the results obtained merely reflect the fact that only one strand of parental DNA enters the progeny – this strand having a different base composition from that of the whole DNA. This would require, however, that there be a corresponding relative increase in contribution to progeny DNA deoxyguanylic acid equal to the deduction in contribution to progeny DNA HMCP. This is not observed.

It is clear that before a vigorous interpretation of these data can be made, a complete material balance of the fate of the DNA nucleotides of the parental phage must be carried out. Current experiments are designed to account for the nucleotides of parental DNA which distributes itself among various fractions of the host cell (intact progeny phage, free DNA, low-molecular-weight fragments and cellular debris).

THE ACID-SOLUBLE RNA OF DEVELOPING AMPHIBIAN EMBRYOS

F. J. Finamore T. Yamada
Rose P. Feldman

Introduction. – Our recent discovery of the existence of acid-soluble RNA in ovarian eggs of *Rana catesbieana*[3] prompted us to investigate the presence and synthesis of this unusual nucleic acid fraction in developing amphibian embryos.

Methods. – Twelve female *Rana pipiens* each received an intraperitoneal injection of 250 μc P^{32} per day for three days followed by two daily injections of 1 mmole of unlabeled phosphate buffer, pH 6.8. Following the last injection of unlabeled buffer, whole pituitaries were administered to induce ovulation. The following day, the P^{32}-labeled eggs were fertilized, and either 800 or 1000 embryos were selected at the various stages of development for the isolation of RNA by the hot sodium chloride extraction procedure. The RNA was separated into acid-soluble and -insoluble fractions and assayed as described previously.[3]

Results. – Table 36 shows that early embryos contain large quantities of acid-soluble RNA, the amount of which decreases markedly after hatching. This decrease takes place apparently at a time when the total quantity of RNA is increasing rapidly in the embryos.

[3]F. J. Finamore and E. Volkin, *J. Biol. Chem.* **236**, 443 (1961).

Table 36. Amount of Acid-Soluble RNA in Embryos

Stage	Total RNA (μg per 10 Embryos)	Acid-Soluble RNA (%)
Two-cell	8.1	89
Gastrula	7.8	89
Early neurula	8.4	82
Late neurula	11.4	84
Hatching	15.9	79
Larva I	24.3	48
Larva II	59.0	27

Figure 63 shows the synthesis of acid-soluble and -insoluble RNA at the various stages of development. Between gastrulation and hatching, acid-soluble RNA is synthesized at a rapid rate, whereas the synthesis of acid-insoluble RNA is sluggish during this time. However, from hatching through larva stage II the rate of synthesis of acid-insoluble RNA increases while that of acid-soluble RNA diminishes. The decrease in amount of acid-soluble RNA, the concomitant increase in P^{32} incorporation into acid-insoluble RNA, and the increase in total RNA per embryo indicate that after hatching the embryo is synthesizing primarily acid-insoluble RNA.

Figure 64 shows the specific activities of the two RNA fractions during development. The specific activity of acid-soluble RNA exceeds several-fold the specific activity of acid-insoluble RNA from the two-cell stage through hatching. However, at some time after hatching, the specific activities of the two RNA fractions become equal and remain that way through the larva stages.

To prove that the acid-soluble RNA fraction from embryos is similar to that found in ovarian eggs, several labeled embryo preparations each were mixed with unlabeled calf liver RNA and hydrolyzed in alkali; and the resulting mononucleotides were separated by column chromatography. Table 37 compares the mononucleotide composition, based on P^{32} counts, of acid-soluble and -insoluble RNA from embryos with that of mature ovarian eggs. No major differences were observed between embryonic and ovarian egg RNA fractions, indicating that the same acid-soluble RNA fraction is present in both eggs and embryos.

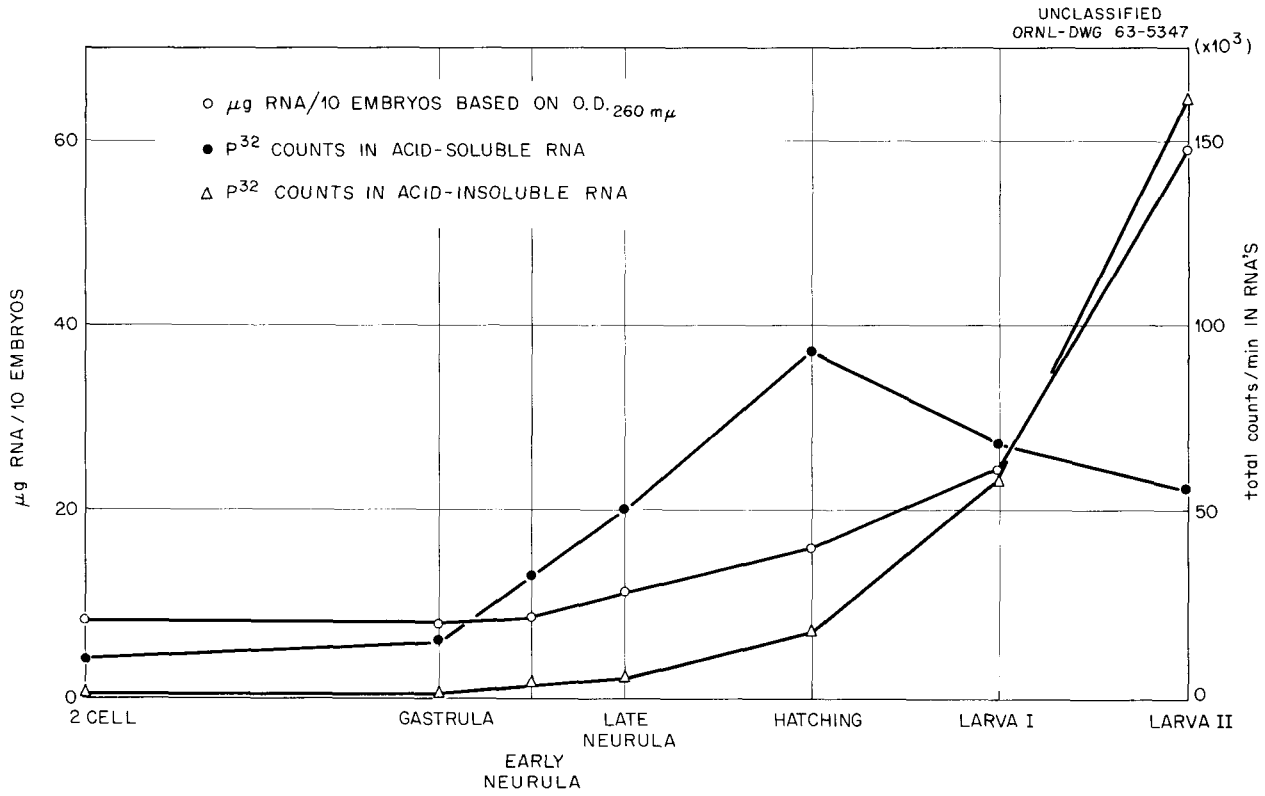

Fig. 63. Synthesis of Acid-Soluble and -Insoluble RNA's.

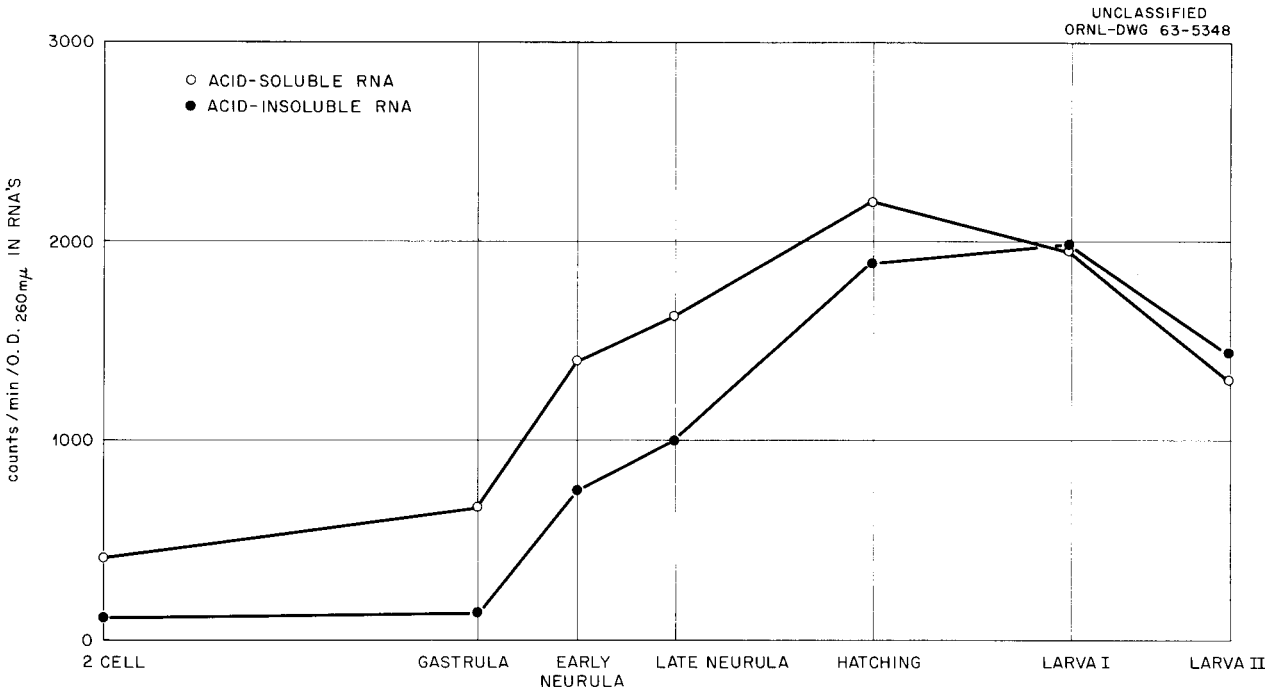

Fig. 64. Specific Activities of Acid-Soluble and -Insoluble RNA's.

Table 37. Nucleotide Composition of RNA's

Acid	RNA Fraction from Embryos (Based on Counts)				RNA Fraction from Ovarian Eggs (Based on Spectrophotometry)	
	Acid-Soluble			Acid-Insoluble	Acid-Soluble	Acid-Insoluble
	Late Neurula	Hatching	Larva I	Larva I		
Cytidylic	26.4	25.6	27.7	34.0	25.3	29.2
Adenylic	28.2	25.8	23.8	17.0	22.8	18.8
Uridylic	18.0	15.3	17.8	10.6	21.3	13.1
Guanylic	27.4	33.3	30.7	38.4	30.5	38.8

SEPARATION OF TRANSFER RNA'S BY CHAIN LENGTHS

C. G. Mead W. E. Barnett

Polynucleotides can be separated according to their degree of polymerization by column chromatography on DEAE-cellulose in the presence of 7 *M* urea.[4] The separation is dependent on the net negative charge of the polynucleotide, and at neutral pH's this is practically independent of base composition.

Ultracentrifugal sedimentation data have indicated that transfer RNA's were homogenous with respect to molecular weights.[5] Of the most successful methods of separating transfer RNA's,

[4]R. V. Tomlinson and G. M. Tener, *Biochemistry* **2**, 697 (1963).

[5]W. A. Klee and G. L. Cantoni, *Proc. Natl. Acad. Sci. U.S.* **46**, 322 (1960).

countercurrent distribution[6] and methylated albumin column chromatography,[7] it is unclear as to what extent molecular weight differences are responsible for the separation because these methods are also sensitive to nucleotide composition and secondary structure. The increased sensitivity in detecting chain length differences provided by the DEAE-cellulose-urea column prompted an examination of chain length differences between transfer RNA's specific for different amino acids.

Transfer RNA was isolated from *Neurospora crassa* by the phenol procedure. The transfer RNA was stripped of amino acids and separated from contaminating ribosomal RNA on a preparative DEAE-cellulose (chloride) column. The purified transfer RNA was then applied to a 1- by 40-cm DEAE-cellulose (formate) column and eluted with a linear gradient of ammonium formate (0.4–0.8 *M*) in the presence of 7 *M* urea. The eluted fractions were assayed for uv absorption and their ability to be charged with phenylalanine, valine, serine, and leucine (Fig. 65).

It is clear that the chromatographic behaviors of the transfer RNA's tested differ on DEAE-cellulose in the presence of urea. If it is correct that the separation on DEAE-cellulose in the presence of urea is dependent on differences in chain length, then it must be concluded that (1) the molecular weight of serine transfer RNA is greater than that of phenylalanine or valine

[6]R. W. Holley and S. H. Merrill, *J. Am. Chem. Soc.* **81**, 753 (1959).

[7]N. Sueoka and T. Yamane, *Proc. Natl. Acad. Sci. U.S.* **48**, 1454 (1962).

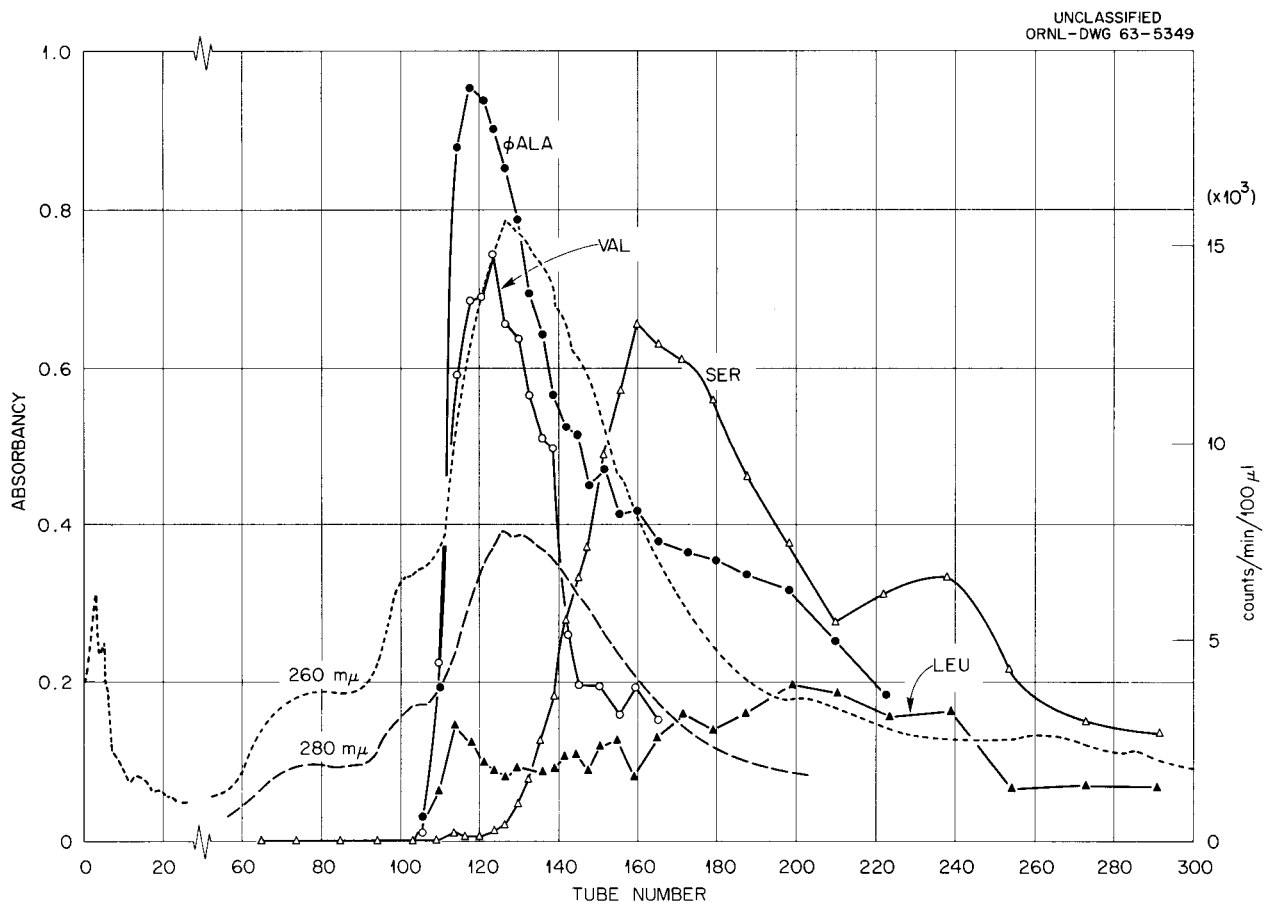

Fig. 65. Ion Exchange Chromatography of Transfer RNA on DEAE Cellulose.

transfer RNA and (2) there is a wide range of molecular weights for leucine transfer RNA. It is planned to measure the chain lengths throughout the chromatogram directly. Additional amino acids will also be tested for their corresponding transfer RNA's in the chromatogram.

USE OF HIGH-MOLECULAR-WEIGHT AMINES FOR PARTITION COLUMNS

J. X. Khym

Introduction. – Saran resin (a copolymer of vinylidene chloride and acrylonitrile) is being tested as an inert carrier[8] to sorb the amine-solvent systems that have been previously described for liquid-liquid extractions of nucleic acid derivatives.[9] The use of water-immiscible amines contained on a column of Saran (or other suitable material) offers possible new chromatographic materials for separations of nucleic derivatives.

Results and Discussion. – "Exchange material" was prepared by mixing 10 ml of 1 *M* JM-T[9] formate dissolved in tridecyl alcohol with 20 g of Saran. Aqueous formate buffers were used to slurry the amine-treated Saran into columns. Initial experiments were carried out with nucleoside mono-, di-, tri-, and tetraphosphates dissolved in aqueous formate buffer solutions. The following observations have been noted.

1. Columns can be reused and reproducible peak positions are obtained if the aqueous formate buffer solutions are saturated with respect to the amine-solvent used on the Saran resin.
2. Slight changes in aqueous salt concentrations change peak positions markedly. This apparently follows from the liquid-liquid extractions[9] wherein extraction coefficients varied sharply with aqueous salt concentrations.
3. Gradient elution techniques have been found to give better elution patterns than those obtained by stepwise elution or elutions at a single salt concentration.

Some initial data typical of those now being collected are shown in Fig. 66. Further experiments will be carried out to explore the use of other amines and solvent systems.

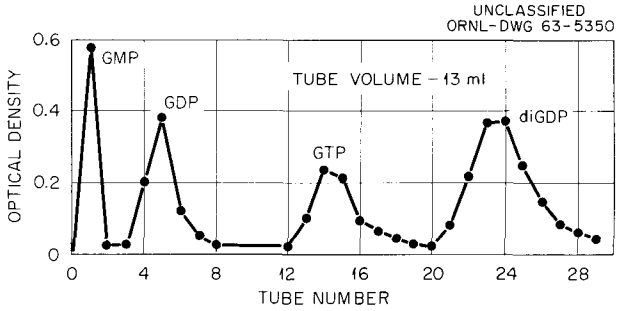

Fig. 66. Separation of Guanosine Phosphates. Exchanger: JM-T formate in tridecyl alcohol sorbed on Saran, 0.2 $cm^2 \times 22$ cm; test solution: GMP (0.6 μmole), GDP (0.7 μmole), GTP (0.5 μmole), P^1P^4 diguanosine 5′-tetraphosphate = diGDP (1.0 μmole) sorbed from 0.1 *M* sodium formate buffer, pH = 4.7; gradient: 0.1 to 0.8 *M* sodium formate, pH 4.7; rate: 0.2 ml/min.

A SEARCH FOR STRUCTURAL CHANGES IN BACTERIOPHAGE DNA AFTER INFECTION

J. L. Fairley

Introduction. – The experiments to be described were undertaken as part of an investigation of the chemical and physical transformations undergone by the DNA of bacteriophage T2 through the cycle of infection and replication in *Escherichia coli*. Particular emphasis was placed on a search for possible forms of DNA differing from the helical duplex found in the mature phage particle.

Methods. – T2 phage highly labeled with P^{32} were used to infect *E. coli* at a multiplicity of 0.5 T2 per bacterium. Aliquots of the suspension of infected cells were quickly chilled at 0, 0.5, 1, 2, 4, 10, 15, and 20 min after infection. The cells were collected by centrifugation and stored until use in a freezer.

The DNA was isolated from each of these preparations by a method essentially that of Mandell and Hershey.[10] Unlabeled carrier phage were first added to provide a high concentration of DNA throughout the extraction procedure and to give an internal reference.

[8] J. X. Khym, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 126.

[9] J. X. Khym, *Biochemistry* 2, 401 (1963).

[10] J. D. Mandell and A. D. Hershey, *Anal. Biochem.* 1, 66 (1960).

The DNA preparations were then examined by two procedures which have been demonstrated to be sensitive to structural and conformational changes in DNA. The first procedure was chromatography on methylated albumin columns prepared according to the modification by Sueoka and Cheng[11] of the original Mandell-Hershey process.[10] Salt and ammonium hydroxide gradients were used to elute the DNA. The second procedure was the distribution of the DNA in the dextran–polyethylene glycol–sodium phosphate system of Albertsson,[12] including both simple distribution and 10-transfer and countercurrent distribution. This system is particularly useful for the differentiation of "native" and "denatured" DNA.

Results and Discussion. – In all experiments involving the chromatography and the phase distribution of the DNA preparations, the behavior of the DNA derived from the original infecting phage (followed by radioactivity measurements) was very similar to that of the DNA provided by the carrier phage (followed by measurements of uv absorption). Such differences as were found were almost surely due to partial fragmentation of the labeled DNA as the direct or indirect result of P^{32} decay.

Obviously no unusual forms of DNA were detected in these experiments. In particular, there was no evidence for the occurrence in infected cells of mainly single-stranded or denatured DNA. Phage DNA which was denatured by heating, added to frozen *E. coli* cells, and then carried through the usual extraction procedure was shown by phase distribution studies to remain chiefly in the denatured form. However, the renaturation in the isolation procedure of such small single-stranded areas as may occur in a mainly double-stranded molecule seems likely.

If unusual DNA structures are formed in the course of phage infection and replication, and at the least structures such as DNA-RNA hybrids must almost certainly occur, the methods described here are apparently not suited for their detection.

[11] N. Sueoka and Ts'ai-Ying Cheng, *J. Mol. Biol.* **4**, 161 (1962).

[12] Per-Åke Albertsson, *Arch. Biochem. Biophys.*, Suppl. 1, **264** (1962).

NUCLEIC ACID METABOLISM IN GERMINATING CORN

N. G. Sansing

Introduction. – Nucleic acid metabolism in germinating corn seedlings has been investigated in an attempt to extend the observations of Volkin, Schwartz, and Jones.[13] The total RNA from the primary root increases through the second day after onset of germination, but then remains constant or even decreases slightly until initiation of secondary roots. During this time, however, the primary root continues to elongate, and there is a several-fold increase in both fresh and dry weight. There is an extensive RNA turnover, however, as evidenced by the rate of uptake and incorporation of P^{32}-labeled phosphate into the RNA of the root.

Results. – In attempts to pulse label with $P^{32}O_4$ and cold chase by addition of an excess of unlabeled phosphate, difficulty was encountered because of the large nucleotide pool size. Using C^{14}-labeled orotic acid, it was found that the activity of the acid-insoluble RNA continued to increase for two days after the labeled material was removed and replaced with unlabeled orotic acid. The endosperm and scutellum were removed from a portion of the seedlings in an attempt to decrease the size of the acid-soluble pool. After three days without the nutritive tissue the plants were placed in labeled phosphate and then removed to unlabeled phosphate for 12 hr. This treatment resulted in a decrease in the amount of label in the acid-soluble pool, but an equivalent decrease was found in the acid-insoluble RNA.

Ribonuclease in the corn seedlings was assayed with a highly sensitive technique adapted from the method of Bollum,[14] using as substrate a purified P^{32}-labeled RNA. Aliquots of a reaction mixture containing the crude enzyme, labeled RNA, and buffer are pipetted onto filter paper disks and washed with cold trichloroacetic acid (TCA) to remove the material rendered acid-soluble by the action of the enzyme. Enzyme activity is determined by following the loss of label from the filter

[13] E. Volkin, D. Schwartz, and M. H. Jones, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1962*, ORNL-3267, pp 118–19.

[14] F. J. Bollum, *J. Biol. Chem.* **234**, 2733 (1959).

paper disk. After three days of germination, the RNase level in the primary root had increased more than 100-fold over that found in the imbibed embryo root.

In preliminary experiments it was found that more than half the RNA in the three-day-old roots was sedimented with the nucleus, whereas less than 10% of the total RNA in the plumule was associated with the nucleus. The base ratio of the nuclear RNA showed a high $G + C$ content, whereas the DNA is high in A and $T[(A + T)/(G + C) = 1.4]$.

Soluble RNA could not be demonstrated in three-day-old roots, although it made up approximately 15% of the total RNA in the plumule. It is not known if the soluble RNA is degraded on rupture of the cell or if the high RNase level results in hydrolysis *in vivo*.

Summary. – From these studies it appears that to have a clearer picture of the nucleic acid metabolism, of roots and plumules, it will be necessary to (1) somehow circumvent the effect of the large nucleotide pool and (2) obtain a thorough understanding of the properties of the tissue nucleases.

Chemical Protection and Enzyme Catalysis

D. G. Doherty
C. F. Holoway[a]
A. J. Speece[a]
J. D. Bales, Jr.
Margaret A. Turner

[a]Research participant.

CHEMICAL PROTECTION

D. G. Doherty Margaret A. Turner
J. D. Bales, Jr.

Introduction. – Research efforts in this area have centered around the examination of new compounds for protective activity and the further exploration of the protection afforded by AET and mixtures of AET and glutathione (GSH) or mercaptoethylamine (MEA) and GSH.[1]

Results and Discussion. – Cysteinylglycine, a component of glutathione, was prepared and found to be ineffective as a radiation protective agent. Guanidoethanol and guanidopropanol, in which the thiol group of mercaptoethyl guanidine (MEG) and mercaptopropyl guanidine is replaced by a hydroxyl group, were synthesized and found to be effective protective agents at 900 r, in $BC3F_1$ mice (LD 50/30 days, 720 r). Guanidoethanol at 1.6 g/kg and 800 mg/kg provided 99% 30-day survival at 900 r and, at the higher dose, gave an LD 50/50 of 1225 r for a relative dose reduction factor (RDF) of 1.7. The propyl compound was less active, 1.6 g/kg giving 97% 30-day survival, but none at 1225 r. Arbutin, a glucoside that might be expected to release hydroquinone, provided no protection against 900 r when administered at 2.4 g/kg prior to irradiation.

A series of experiments was performed to determine the extent to which mice could be protected against multiple lethal doses of x radiation by AET or mixtures of AET/GSH and MEA/GSH. Mice treated with 160 mg/kg AET (as MEG intraperitoneally) and given 900 r all survived 30 days. Repetition of this process yielded 80% 30-day survivors which were again treated and irradiated. No animals survived the third dose of radiation. If 60 days was allowed to elapse between treatments, all survived the first, 95% survived the second, and none the third. Thus, for this dose of AET, 1800 r was the maximum the mice could tolerate. At 360 mg/kg AET and a 30-day interval, the survival was comparable to the above experiment with a 60-day recovery for the first and second irradiation and, in addition, half the animals survived a third 900 r. Using the same protocol and treating the mice with mixtures of AET/GSH or MEA/GSH plus 1200 r at 30-day intervals gave 95% survival after the second dose and 50% after the third dose for a total of 3600 r. Multiple doses of 1400 r gave 65% survival after the second and 35% after the third irradiation for a total of 4200 r. These results indicate that the addition of GSH to either AET or MEA increases the protection obtained at supralethal dose levels.

As an additional measure of the effectiveness of the mixtures, the incorporation of Fe^{59} in the spleen[2] of treated animals was measured. As

[1]D. G. Doherty, J. D. Bales, Jr., and M. A. Turner, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 129.

[2]L. H. Smith and T. W. McKinley, Jr., *ibid.*, p 72.

can be seen from Fig. 67, at 900 r there was little difference between 360 mg/kg AET and 480 mg/kg AET/1.6 g/kg GSH. At 1400 r the mixture was just as effective as it was at 900 r and closely resembled the 450-r control. These results are in accord with previous measurements of spleen recovery using cell-counting techniques, both methods showing a peak in spleen activity between 11 and 16 days.

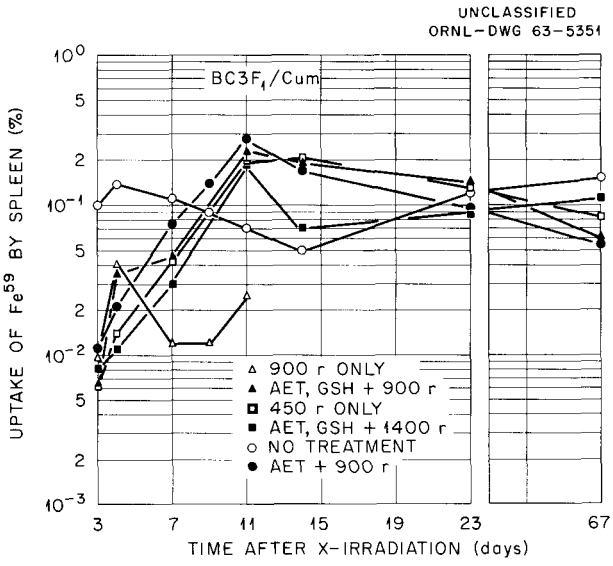

Fig. 67. Fe^{59} Incorporation in the Spleen.

PHOSPHORUS-CONTAINING PROTECTIVE AGENTS

C. F. Holoway D. G. Doherty

Recently, sodium hydrogen cysteamine *S*-phosphate,[3] aminoethyl ethanethiophosphonate,[4] and *S*-β-*N*-methylaminoethyl hydrogen phosphorothioate[5] were reported to possess marginal protection against radiation. On the basis of the preceding reports and because of the central role of phosphates in biological systems, further exploration of phosphorus and thiophosphorus compounds of such types as $R_1R_2N(CH_2)_nSP(X)R_3R_4$ and $R_1S(CH_2)_nNHP(X)R_2R_3$ (where R_1, R_2, R_3, and R_4 = H, OH, alkyl and/or alkoxy, X = O or S, and n = 0 to 4) was felt promising. Development required a literature search for suitable syntheses and exploratory laboratory work prior to the synthesis of a variety of prototypes.

The following methods were examined in the laboratory briefly: (1) aminophosphonates from bromalkylphthalimides and bromalkylamine hydrobromides, (2) aminoalkylphosphorothioates from bromalkylamine hydrobromides, (3) guanidophosphates from arylphosphoryl halides, and (4) aminoalkylphosphorothioates from sodium phosphorothioates and from phosphoryl halides. It was concluded that thermal isomerizations of the Arbusov type, followed by HBr hydrolysis of the alkyl esters, though classical, lead to complex side reactions and low yields, dependent upon conditions used. Thus, the use of bifunctional molecules, such as bromalkylamine hydrobromides with trialkyl phosphites, needs further study. Ionic reaction, as carried out by the Ackerfeldt method, yields a product analyzing correctly, but the exact structure of the product is at present in doubt, one factor being that of isomerization and another that of the existence and lability of the P–S bond. Guanidophosphates, as prepared by Cramer *et al.*,[6] seem promising, provided benzyl or other protective group labile to hydrogenolysis is used. The method cannot be extended to include preparation of isothioureidophosphates, though it can be used to prepare isoureidophosphates and may be applicable to the preparation of phosphoramidates, depending on lability of the P–N bond, the latter type of compound not yet having been examined. The use of sodium phosphorothiolates and thionates, and of phosphoryl halides, to prepare both intermediates and desired amino (thio) phosphates offers both versatility and simplicity. Methanol is a compromise solvent that dissolves both salts (sodium bromide and sodium organophosphorothioates) and nonpolar intermediates such as ethylenebromide for interaction, salt being eliminated by benzene extraction of evaporated residues.

The following compounds were synthesized and are being evaluated: sodium hydrogen cysteamine

[3] S. Ackerfeldt, *Acta Chem. Scand.* 13, 1479 (1959).

[4] B. Hansen and B. Söbro, *Acta Radiologica* 56, 141 (1961).

[5] D. G. Doherty, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 129.

[6] F. Cramer and A. Vollmar, *Chem. Ber.* 91, 919 (1958).

S-phosphate; *O,O*-diethylphosphoramidate; diethylpyrrolidinylphosphate (cyclized 4-bromutylamine) phosphate intermediate; and *O,O,S*-triethylphosphorodithioate. Additional compounds are being synthesized as intermediates and for testing.

HISTOCHEMICAL DISTRIBUTION OF LYSOZYME ACTIVITY IN ORGANS OF IRRADIATION CHIMERAS

A. J. Speece

Previous reports of lysozyme activity in animal tissues have been based on the reaction between supernatants from tissue homogenates with suspensions of *Micrococcus lysodeikticus* or cell derivatives as substrate.[7,8] A method was devised for demonstrating the activity of animal cells as they are situated in tissue sections of the various organs. This method was used to study the lysozyme distribution in normal and x-irradiated normal and chimeric mice, killed on the ninth day after irradiation. The animals used in this study were of the same strain and were treated as described by Suu.[9]

Microslides were coated with a film of Worthington *M. lysodeikticus*. Cryostat sections of fresh frozen organs were placed on these slides and incubated for a timed interval. These preparations were then stained and differentiated to produce a visible difference between bacteria that were affected and those not affected by lysozyme action from the apposed animal cells. This method depends on diffusion of lysozyme from the section to the bacteria; hence the most active cells produced a notable reaction in about 30 sec, with lateral diffusion of 5 to 10 μ. Less active cells required from 1 to 5 min to give a similar reaction, and diffusion prevented precise localization. By this method it was possible to identify certain cells directly, while others were identified by association with the larger structures they constituted.

Lysozyme distribution in the normal mouse can be summarized as follows:

1. *Very active:* polymorphonuclear leukocytes; Paneth cells; and marrow cells except megakaryocytes, macrophages, erythrocytes, and some lymphocytes.
2. *Moderately active:* lymphocytes, large, more active than small; proximal tubule and glomerulus, the latter much less active; acinar cells of pancreas; lung tissue; Brunner's glands; and lamina propria of intestine and colon.
3. *Little or no activity:* liver, thyroid, stomach, testis, salivary gland, all muscle tissue, thymus, adrenal, and columnar epithelium of gastrointestinal tract.

Changes in lysozyme activity in irradiated mice may be summarized as follows: An increase in lysozyme activity was noted in the proximal tubules and glomeruli. Acinar cells of the pancreas lost most of their activity. In the radiation chimeras the leukocytes showed increased activity, as well as numbers, and more activity was noted in the lamina propria of the intestinal tract. All other changes noted could be attributed to the transplantation of foreign cells and proliferation and redistribution of the cellular elements of the host animals.

Acid phosphatase tests by the α-naphthyl phosphate–pararosaniline method recommended by Barka[10] were done on the tissues to superimpose the two techniques in the same sections. It is well established that the acid phosphatase activity is associated with the lysosome,[11,12] and it is suspected that this structure also gives rise to lysozyme. The distribution of activity of these two enzymes indicates that they are probably not produced by the same structures at the same time or in the same cells. The acid phosphatase activity was useful in identification of macrophages and proximal tubules and thus assisted in determining which cells produced the lysozyme activity.

[7] P. Jolles, *The Enzymes* (ed. by P. D. Boyer, H. Lardy, and K. Hyrbäck), vol IV, 2d ed., p 432, Academic Press, New York, 1960.

[8] Vu-Thi Suu, C. C Congdon, and A. L. Kretchmar, *Proc. Soc. Exptl. Biol. Med.* **113**, 481 (1963).

[9] Vu-Thi Suu, this report.

[10] T. Barka, *Nature* **187**, 248 (1960).

[11] C. Duve, *Subcellular Particles*, American Physiological Society, Washington, D.C., 1959.

[12] E. Essner and A. Novikoff, *J. Biophys. Biochem. Cytol.* **9**, 773 (1961).

ACUTE TOXICITY OF CHEMICAL CARCINOGENS

D. G. Doherty Francis Hacker

The acute toxicity of representative members of the major classes of chemical carcinogens is of interest in experiments with AET protection and bone marrow transplantation recovery. Chemical carcinogens often damage the same organ systems that show acute injury after lethal whole-body irradiation.

When a chemical carcinogen in acute lethal doses causes morbidity through bone marrow or lymphatic tissue injury, then marrow transplantation might have a recovery effect on the disease. Marrow transplants might also have an effect on tumor production by the chemical. There is a well-known experiment of prevention of radiation-induced thymic lymphosarcoma by bone marrow therapy.

Protection against chemical damage has already been shown for lethal doses of certain alkylating agents. These agents form one of the classes of chemical carcinogens.

Tables 38 to 40 show the intraperitoneal, oral, and subcutaneous toxicity in mice of 9,10-dimethyl-1,2-benzanthracene (DMBA) in olive oil. Toxicity of the olive oil vehicle alone was not observed.

A preliminary attempt to show protection with AET orally (16 mg) and recovery with 40×10^6 isologous bone marrow cells (IBM) intravenously on DMBA toxicity is given in Table 41. No protection or recovery was observed in these experiments. AET would not be expected to affect DMBA toxicity.

In a separate experiment, where mice were examined at 7- and 14-day intervals after DMBA and IBM, there was marked recovery of the marrow in the IBM group compared to the DMBA control.

Table 38. Acute Intraperitoneal Toxicity of DMBA in Olive Oil

Dose (mg)	Strain[a]	Number of Mice	Sex	Number Dead at Day				Mortality (%)
				7	14	21	28	
2	LAF_1	5	F	0	0	0	3	60
3	LAF_1	10	F	6	6	8	8	80
4	LAF_1	10	F	0	9	9	9	90
5	LAF_1	8	F	0	8	8	8	100
6	LAF_1	15	F	0	13	14	14	93
9–30	LAF_1	35	F	33	35	35	35	100
4.8	$1C3F_1$	40	M & F	0	36	37	38	95
3.6	$B6D2F_1/J$	20	F	1	20	20	20	100
4.8	$B6D2F_1/J$	20	M	0	19	19	20	100

[a]38 to 42 weeks of age.

Table 39. Acute Oral Toxicity of DMBA in Olive Oil

Dose (mg)	Strain	Number of Mice	Sex	Number Dead at Day				Mortality (%)
				7	14	21	28	
2.4–7.2	$1C3F_1$/Cum	15	F	0	0	0	0	0
10–15	$B6D2F_1/J$	10	F	0	0	0	0	0
20	$B6D2F_1/J$	5	F	5	5	5	5	100

Table 40. Subcutaneous Toxicity of DMBA in Olive Oil

Dose (mg)	Strain[a]	Number of Mice	Number Dead at Day				Mortality (%)
			7	14	21	28	
4	$1C3F_1$/Cum	5	0	0	0	1	20
6	$1C3F_1$/Cum	5	0	2	3	4	80
8	$1C3F_1$/Cum	5	0	4	5	5	100
10	$1C3F_1$/Cum	5	0	5	5	5	100
2.4–3.6	$B6D2F_1$/J	10	0	0	0	0	0
4.8	$B6D2F_1$/J	5	0	1	1	1	20

[a]All mice were male.

Table 41. DMBA Intraperitoneally (4.8 mg) in Olive Oil

Treatment	Strain	Number of Mice	Sex	Number Dead at Day				Mortality (%)
				7	14	21	28	
Olive oil ip (0.4 ml)	$1C3F_1$	40	M & F	0	0	0	0	0
DMBA	$1C3F_1$	40	M & F	0	36	37	38	95
AET + DMBA	$1C3F_1$	40	M & F	6	38	40	40	100
AET + DMBA + IBM	$1C3F_1$	10	M	0	10	10	10	100
DMBA + IBM	$1C3F_1$	10	M	0	10	10	10	100
Olive oil ip (0.4 ml)	$B6D2F_1$/J	20	M	0	0	0	0	0
DMBA	$B6D2F_1$/J	20	M	0	19	19	20	100
AET + DMBA	$B6D2F_1$/J	20	M	3	16	18	18	90
AET + DMBA + IBM	$B6D2F_1$/J	20	M	2	20	20	20	100
DMBA + IBM	$B6D2F_1$/J	20	M	1	19	19	20	100

Acute toxicity studies and AET protection have also been carried out on butter yellow and 2-aminofluorene (2-AF). Protection was not observed in these preliminary experiments.

PATHOLOGIC CHANGES IN MICE GIVEN LETHAL DOSES OF CHEMICAL CARCINOGENS

C. C Congdon[13]

Preliminary observations have been made on mice exposed to acute toxic amounts of butter yellow, 2-aminofluorene (2-AF), and 9,10-dimethyl-1,2-benzanthracene (DMBA) to determine the major modes of death. This study, carried out in collaboration with D. G. Doherty, is intended to provide information that might be used as a basis for protection and recovery measures against acute carcinogen toxicity and chemical tumor induction. In addition, assessment of pathologic changes is one of the methods of measuring protection and recovery effects. The project is a part of the Biology Division's new program to investigate action of chemical carcinogenic and mutagenic agents on a wide variety of biological systems.

[13]Mammalian Recovery Group.

DMBA in olive oil administered intraperitoneally produced a severe generalized peritonitis. Extreme bone marrow and spleen damage, as well as injury to pancreas, adrenal, thymus, and lymphatic tissues were noted. In one experiment, in which isologous bone marrow was given three days after DMBA, there was marked regeneration of the recipients' bone marrow.

Autopsy studies after oral and subcutaneous injection of DMBA have not been completed.

Butter yellow in olive oil produced marked spleen, thymus, liver, and kidney damage, and slight bone marrow injury. Death was attributed to liver and kidney failure. In mice surviving the toxic dose, there was extreme regenerative hyperplasia of erythropoietic elements in the spleen red pulp and regeneration of the tubular epithelium in the kidney. The liver recovered from its severe degenerative fatty infiltration.

With 2-AF in olive oil given intraperitoneally, two animals in one experiment showed generalized peritonitis, but it was not seen in several other mice given the chemical at the same time. Slight injury to marrow was observed as well as necrosis of the surface of the pancreas. Extreme atrophy of the spleen was present, with toxic changes in liver and kidney. Thymus and lymphatic tissue damage also developed. The most common mode of death for this compound was not established. One surviving animal that lived 44 days had at autopsy a lymphosarcoma of the thymus that was widely disseminated throughout many organs.

Enzymology

G. D. Novelli

Protein Synthesis

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Hormonal Control

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Mammalian Cell Regulation

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Energy Metabolism

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Research in the Enzymology Group can be considered in the following categories: (1) enzyme mechanisms in protein synthesis and associated nucleic acid synthesis, (2) hormonal control of protein synthesis, (3) mammalian cell regulation, and (4) energy metabolism.

Protein Synthesis

The reports on protein synthesis describe our efforts to study amino acid incorporation and protein synthesis in a number of cell-free systems (i.e., systems prepared from *Escherichia coli*, *Clostridium pasteurianum*, rat liver, and maize). Because protein synthesis seems to be dependent on an RNA made by a DNA-dependent RNA polymerase, we are attempting to prepare this enzyme on a large scale to be used in all the cell-free systems currently under investigation. In the work with the maize system, we hope to ultimately be able to synthesize a specific esterase whose synthesis is under genetic control. Thus, while we are continuing to characterize the cell-free systems from maize tissues, we are simultaneously purifying the pH 7.5 esterases from maize to prepare antibodies that will be used as reagents to isolate and characterize any esterases we may synthesize in the cell-free systems.

The regenerating rat liver gives us a tissue that is suddenly called upon to synthesize new enzymes and offers the opportunity to study the relation between the synthesis of new RNA and new protein. By density gradient analyses of the ribosomal population, we hope to collect only those polysomes actively engaged in protein synthesis and to exclude from our preparations those that are inactive.

As a continuing study of the mechanism of protein synthesis, we have continued our studies with S-RNA to determine whether the code is universal and whether amino acid activating enzymes will make mistakes by placing the wrong amino acid on heterologous S-RNA.

Finally, in anticipation of work with small amounts of purified protein that may be isolated from our systems, we are attempting to develop a "molecular balance" that will enable us to determine molecular weights of small amounts of polymerase by studying the behavior of surface monolayers of films of the polymers when they are compressed.

A SOLUBLE AMINO ACID INCORPORATING SYSTEM FROM RAT LIVER

Hideko Kaji G. D. Novelli
Akira Kaji

Introduction. – Previously we reported that a soluble extract from *Escherichia coli* actively incorporates C^{14}-labeled amino acids into a protein fraction without addition of the ribosomal components.[1,2] In an attempt to determine the general biological significance of this soluble incorporating system, we chose to study rat liver to examine the presence of a similar system. Quickly isolated livers were homogenized and centrifuged for 20 min at 20,000 × *g* and at 32,000 × *g*. The supernatant obtained was further centrifuged at 85,000 × *g* for 2.5 hr and was treated with streptomycin sulfate. This supernatant was treated with protamine, and the precipitate was eluted by ammonium sulfate. The eluate was fractionated by ammonium sulfate to obtain three fractions, I, II, and III, which were dialyzed. In some cases the supernatant obtained after addition of streptomycin was brought to 75% saturation with $(NH_4)_2SO_4$, and the precipitate was dialyzed.

Results and Discussion. – Radioactive arginine was incorporated into the protein fraction when fraction II was incubated in the presence of ATP, an ATP generator, amino acids, and S-RNA. Incorporation was dependent on S-RNA and the energy generator, but not on the presence of GTP. The S-RNA prepared from *E. coli* could partially replace rat liver S-RNA. Pancreatic RNase completely inhibited the incorporation. Deletion of the amino acid mixture depressed the incorporation of arginine slightly. Puromycin, at the level of 2×10^{-4} *M*, caused 50% inhibition. The incorporating activity was found in fractions I, II, streptomycin precipitate, and the protamine supernatant. Evidence that this incorporating activity

[1] Akira Kaji and Hideko Kaji, *Federation Proc.* **22**, 302 (1963).

[2] Akira Kaji, Hideko Kaji, and G. D. Novelli, *Biochem. Biophys. Res. Communs.* **10**, 406 (1963).

of fraction II does not depend on the traces of ribosomes was obtained from a study of the sedimentation behavior of this fraction, which contained only a trace of an 18 S (Svedberg unit) component as the most rapidly sedimenting material. Furthermore, the activity was retained in the supernatant upon 2 hr centrifugation at 105,000 × *g*. The sucrose density gradient centrifugation of the streptomycin supernatant showed that the peak of incorporating activity corresponded to approximately 10 S. The incorporated activity by sucrose density gradient centrifugation showed that the peak of incorporated radioactivity was approximately 8 S. The treatment by proteolytic enzymes made the radioactive product acid-soluble. Of the 17 labeled amino acids tested, only arginine was incorporated as much as that incorporated by the ordinary ribosome-supernatant system when fraction II was used. However, glycine, glutamic acid, methionine, and tryptophan, as well as arginine, were incorporated by streptomycin supernatant. This rat liver soluble system is remarkably similar to the *E. coli* soluble system. Evidence from both systems strongly suggests that the soluble incorporating system is somehow related to the conventional particle-supernatant, protein-synthesizing system.

A STUDY OF PHAGE-INDUCED PROTEIN SYNTHESIS IN *ESCHERICHIA COLI*

J. V. Schnell G. D. Novelli

Introduction. – Infection of a susceptible bacterial cell by a bacteriophage usually results in the release of many newly synthesized viral particles upon the lysis of the host. Since the infective nucleic acid apparently directs the biosynthetic processes of the host organism to produce large amounts of viral protein and nucleic acid, phage-infected bacterial systems should provide a valuable tool for the study of protein biosynthesis. The discovery of bacteriophage which contains infective RNA[3] provides a unique opportunity to study the relation of viral RNA to protein synthesis. This research project will involve a study of the biosynthesis of the RNA-primed, RNA-synthesizing enzyme(s) appearing upon infection of a bacterium by an RNA-containing phage.

Results and Discussion. – Bacteriophage μ-2, an RNA-containing phage, and host strain *E. coli* Hfr_1 were obtained from the Institute of Microbiology of the University of Milan, Italy. Conditions for the growth of this bacterium and its virus have been studied, and it is now possible to obtain lysis of the host in liquid culture and on an agar medium. Quantitative assay for phage has been achieved by plaque counts of plates prepared by the agar layer technique.[4] A 30-liter culture of the virus was prepared, with a yield of 4×10^{14} plaque-forming units. The phage isolated from this culture will be used as an inoculum for future experiments and as a source material for the isolation of viral RNA. Purified phage RNA will be characterized and tested as a primer for the study of the biosynthesis of the phage-induced RNA-synthesizing enzyme(s).

Weissmann *et al.*[5] have demonstrated that infection of *E. coli* Hfr_1 with the RNA phage MS-2 leads to the appearance of an RNA-primed, RNA-synthesizing enzyme apparently absent from uninfected cells. This enzyme has been called "RNA synthetase" to distinguish it from other RNA polymerase systems. The phage μ-2 will be used in an attempt to demonstrate the induction of this enzyme to obtain a system for the investigation of the biosynthesis of RNA synthetase. The appearance of this enzyme will be assayed by measuring the ability of extracts of infected cells to incorporate C^{14}-UTP into RNA. Attempts will be made to achieve cell-free synthesis of RNA synthetase by extracts of normal cells in the presence of viral RNA. For these experiments, a measure of the incorporation of C^{14}-histidine into protein should be useful as an assay system. Histidine is the amino acid of choice since it has been reported that the protein of the coat of phage f-2 contains no histidine.[6] Proposed experiments on the system will be designed to obtain information relating to the following questions: (1) Does viral RNA function as messenger RNA for the biosynthesis of RNA

[3]T. Loeb and N. D. Zinder, *Proc. Natl. Acad. Sci. U.S.* **47**, 282 (1961).

[4]M. H. Adams, *Bacteriophages*, Interscience, New York, 1959.

[5]C. Weissmann, L. Simon, and S. Ochoa, *Proc. Natl. Acad. Sci. U.S.* **49**, 407 (1963).

[6]D. Nathans *et al.*, *Proc. Natl. Acad. Sci. U.S.* **48**, 1424 (1962).

synthetase? (2) Does phage-induced protein synthesis follow the same metabolic scheme as that in the normal cell? (3) Does the RNA synthetase produce viral RNA?

AMINO ACID INCORPORATION BY CELL-FREE EXTRACTS OF *CLOSTRIDIUM PASTEURIANUM*

R. D. MacElroy H. D. Peck, Jr.
G. D. Novelli

Introduction. – A new project has been initiated to study the biosynthesis of the iron-containing electron transport protein, ferredoxin.[7] This protein has a low molecular weight (5,000–10,000), and its amino acid composition is quite different from that of other proteins. The ferredoxin molecule lacks the amino acids histidine, arginine, methionine, leucine, and tryptophan, but does contain comparatively large amounts of aspartic acid, alanine, valine, and isoleucine.[8] The protein can be easily purified by utilizing its property of being soluble in 50% acetone. It is intended to utilize the anomalous amino acid composition and solubility property to develop a protein-synthesizing system in which amino acid incorporation into a single protein can be readily studied. As a first step toward this goal, an attempt is being made to obtain a cell-free amino acid incorporating system from *C. pasteurianum.*

Results and Discussion. – Cell-free extracts of *C. pasteurianum* have been prepared using the French pressure cell and particles, presumably ribosomes isolated by high-speed centrifugation. The crude extract incorporates C^{14}-leucine into hot trichloroacetic acid labile (S-RNA) and stable (protein) components. After removal of the particles, the soluble protein no longer incorporates leucine that is stable to hot trichloroacetic acid; however, recombination of the particles with the soluble proteins restores this activity. The requirements and conditions for optimum activity are now being ascertained to facilitate the proposed studies on the biosynthesis of ferredoxin.

[7] L. E. Mortenson, R. C. Valentine, and J. E. Carnahan, *Biochem. Biophys. Res. Communs.* **7**, 448 (1962).

[8] B. B. Buchanan, Walter Lovenberg, and J. C. Rabinowitz, *Proc. Natl. Acad. Sci. U.S.* **49**, 345 (1963).

A LARGE-SCALE FACILITY FOR THE CULTIVATION OF MICROORGANISMS AND FOR THE PREPARATION OF BIOLOGICALLY ACTIVE MATERIALS

E. F. Phares H. D. Peck, Jr.
Mary V. Long G. D. Novelli

Introduction. – The current work of a number of groups in the Biology Division is dependent upon their having access to large amounts of microorganisms from which a number of biologically active materials can be prepared. Some of the materials are the DNA-dependent RNA polymerase, DNA polymerase, polynucleotide phosphorylase, RNA synthetase, ribonuclease, β-galactosidase, polysomes, and S-RNA. A facility for the cultivation of a variety of microorganisms has been designed and is under construction.

Results and Discussion. – The pilot plant is presently equipped with a New Brunswick fermentor consisting of six 14-liter cylindrical vessels set up with a variable-speed agitator, controlled airflow, and foam control. Thus, six variables can be tested simultaneously. There are two Biogens for continuous culture of microorganisms, one with a capacity of 40 to 80 liters and a larger one with a capacity of 100 to 200 liters. In addition, there is a 100-gal vat-type fermentor. For harvesting cells, there are four air turbine-driven Sharples centrifuges, one industrial-type Sharples, and one Westphalia disk-type separator. A number of different devices for breaking cells are available. These are (1) a constant-pressure French cell with a capacity of 40 ml, (2) a Cullen cell homogenizer capable of producing 1 liter of extract per min, (3) an Eppenbach micromill that breaks cells with the use of glass beads and has a capacity of 16 gal, and (4) a 9- and a 10-kc Raytheon sonic oscillator and a 20-kc Biosonik apparatus that can be operated continuously. Thus we have the capability of producing cells of microorganisms from a few grams to several kilos and the corresponding facilities to produce extracts on almost any scale.

In the future this pilot plant will have a large cold room adjacent that will permit the large-scale fractionation of cell extracts. A large pan-type lyophilizer and a filter press will also be added.

FURTHER STUDIES ON THE PURIFICATION OF THE DNA-DEPENDENT RNA POLYMERASE FROM *ESCHERICHIA COLI*

L. Rosen A. Marie Manning
G. D. Novelli

Introduction. – We are continuing our investigation of a suitable procedure for the large-scale purification of the DNA-dependent RNA polymerase from *Escherichia coli.*[9]

Results and Discussion. – We have worked out a purification procedure which can reproducibly yield RNA polymerase that is 100-fold purified with a yield of 60%. The procedure involves removal of ribosomes from a French cell extract by centrifugation at 78,000 × *g* for 4 hr. The enzyme is precipitated by treating the supernatant with protamine sulfate, and after extensive washing of the protamine precipitate, the enzyme is eluted with 0.5 *M* sodium succinate at pH 6.0. Then the enzyme is precipitated by bringing the solution to 0.4 saturation with ammonium sulfate. This precipitate is then washed successively with 0.60, 0.50, and 0.45 saturated $(NH_4)_2SO_4$, and the enzyme is recovered by two washes with 0.4 saturated $(NH_4)_2SO_4$. Enzyme thus prepared is stable when stored in liquid nitrogen. RNA synthesis by such a preparation is DNA dependent, with different preparations of DNA giving varying activities. Calf thymus DNA (from Worthington Co.) and *E. coli* DNA prepared here give equal priming activities, whereas calf thymus DNA prepared by F. J. Bollum of this division has about twice the priming activity of the former DNA's. The enzyme catalyzes the net synthesis of RNA and makes about three times as much RNA as the amount of DNA used as primer. At this stage of purification, the enzyme will incorporate 3 micromoles of nucleotide per milligram per hour. Assuming that all nucleotides are used randomly, 1 mg of enzyme can make 4.2 mg of RNA. The yield of enzyme in the above purification procedure is 30 mg per 100 g of cells. Under our present operating conditions, we can work up 1000 g of cells per week to yield 300 mg of enzyme.

We shall continue to stockpile enzyme at this stage of purification until we have accumulated enough to warrant continuation of more purification steps.

[9]L. Rosen, A. Marie Manning, and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 134.

BINDING OF AMINO ACYL–S-RNA TO RAT LIVER POLYSOMES

Piero Cammarano Giovanni Giudice
G. D. Novelli

Introduction. – In a previous report[10] we noted that much of the S-RNA of mouse liver was bound to ribosomes. We subsequently found that a similar situation existed in rat liver. During a study of the function of polysomes in amino acid incorporation, we noted that the C^{14}-labeled leucine was first incorporated into protein by the heavier polysomes and subsequently appeared with the 70 S ribosome or monomeric units.[11] We wondered what happened to the C^{14}–amino acid bound to S-RNA while the former process was in progress.

Results and Discussion. – When measuring the incorporation of a C^{14}-labeled amino acid into protein in an *in vitro* system using the filter paper disk procedure, one can obtain a value for amino acid incorporated into protein and for amino acid bound to S-RNA by noting the difference into total counts between identical disks treated with cold trichloroacetic acid (TCA) and those treated with both hot and cold TCA. The latter gives the value for amino acid incorporated into protein, and the difference between the former and the latter is the value for amino acid bound to S-RNA.

Ribosomes were incubated with C^{14}-leucine under conditions of protein synthesis and at 5, 15, and 30 min after incubation samples were analyzed by zonal centrifugation in a sucrose density gradient. Each fraction was tested for amino acid incorporated into protein and amino acid bound to S-RNA. After 5 min of incubation, the radioactivity of the ribosome-bound amino acyl–S-RNA is distributed in peaks, covering the particle size from 67 to 200 S. The greatest amount is in association with aggregates of sedimentation coefficient greater than 96 S, with a

[10]Piero Cammarano and G. D. Novelli, *ibid.*, p 135.

[11]Piero Cammarano, Giovanni Giudice, and G. D. Novelli, *Biochem. Biophys. Res. Communs.*, in press.

major peak corresponding to aggregates of sedimentation constant at 135 and 154 S; only a small amount of radioactivity is found associated with the monomer pool, whose specific activity is the lowest one. After 15 min of incubation, there is a relative reduction in the radioactivity associated with the heavy aggregates, paralleled by a corresponding increase of the radioactivity included in the region of aggregates of sedimentation constant of 135 S. An increase of radioactivity in the region of the monomer pool is also observable. After 30 min of incubation, the major peak of radioactivity is associated with the monomers, followed by the dimer and the trimer peaks respectively.

Taken as a whole, these results indicate that under conditions of protein synthesis the amino acyl–S-RNA is largely bound to the polysomes and follows their progressive monomerization during amino acid incorporation. This fact strongly suggests that the 67 S units are the sites of binding of amino acyl–S-RNA to polysomes, although partial attachment to the inter-ribosomal RNA cannot be excluded.

EFFECT OF ACTINOMYCIN D ON THE SYNTHESIS OF DNA POLYMERASE IN HEPATECTOMIZED RATS

Giovanni Giudice G. D. Novelli

Introduction. – From the work of Bollum and Potter,[12,13] it is known that the enzymatic activity of DNA polymerase is very low in normal rat liver. After partial hepatectomy this activity begins to increase 18 hr after the operation, increases exponentionally up to 24 hr, then continues to increase at a slower rate, reaching a maximum at about 30 hr.

Bollum *et al.*[14] have shown that whole-body x irradiation is effective in suppressing this rise in DNA polymerase if given early in the regeneration period. This, and other reports, suggests that a specific RNA might have to be synthesized early in regeneration in order to effect the noted rise in DNA polymerase. It was recently shown that actinomycin D inhibits the DNA-dependent RNA polymerase.[15] It occurred to us that the injection of actinomycin D after hepatectomy might reveal whether a DNA-dependent synthesis of RNA is required for the increase in DNA polymerase.

Results and Discussion. – Male Sprague-Dawley albino rats, weighing from 140 to 180 g, were subjected to 70% hepatectomy under ether anesthesia. A suspension of 70 μg of actinomycin D in 0.1 ml of 1,2-propanediol was injected intraperitoneally at 3, 8, 12, 16, and 20 hr after hepatectomy. All animals were sacrificed 30 hr after hepatectomy, and the DNA polymerase activity was measured according to method III of Bollum and Potter.[13] Actinomycin injection given 3 and 8 hr after hepatectomy almost completely inhibited the expected rise in DNA polymerase activity. If the actinomycin injection is delayed for 12 hr or longer after hepatectomy, there is a much lower inhibition of DNA polymerase. If actinomycin injection is delayed until 20 hr after hepatectomy, no inhibition is observed.

Therefore, with actinomycin D administration, as well as with x irradiation, a period of sensitivity exists which corresponds to the early stages of regeneration. Our results suggest the existence of a period of intense RNA synthesis up to 12 hr after hepatectomy. The administration of actinomycin during this period prevents this synthesis and therefore the subsequent synthesis of DNA polymerase. We also measured the effect of actinomycin injection on total protein synthesis of the liver by measuring the radioactivity of liver proteins following a short exposure to C^{14}-leucine. There was essentially no difference in C^{14}-leucine incorporation between the controls and the actinomycin-treated animals. Thus it seems the actinomycin inhibits only the synthesis of new proteins that require the synthesis of a new RNA.

SUCROSE DENSITY GRADIENT ANALYSIS OF REGENERATING RAT LIVER RIBOSOMES

Giovanni Giudice Piero Cammarano
G. D. Novelli

Introduction. – Evidence has been presented for an enhancement of rat liver protein synthesis

[12] F. J. Bollum and V. R. Potter, *J. Biol. Chem.* **233**, 478 (1958).

[13] F. J. Bollum and V. R. Potter, *Cancer Res.* **19**, 561 (1959).

[14] F. J. Bollum *et al.*, *Cancer Res.* **20**, 138 (1960).

[15] J. Hurwitz *et al.*, *Proc. Natl. Acad. Sci. U.S.* **48**, 1222 (1962); I. H. Goldberg, M. Rabinowitz, and E. Reich, *Proc. Natl. Acad. Sci. U.S.* **48**, 2094 (1962).

in vivo and *in vitro* following partial hepatectomy.[16] Since it has been recently proposed that the active sites of protein synthesis are ribosomal aggregates named "ergosomes" or polysomes,[17] it seemed worthwhile to investigate the pattern of distribution in a sucrose density gradient centrifugation of the ribosomal population at various stages of rat liver regeneration as compared with the ribosomes of normal rat liver.

Results and Discussion. – Ribosomes were prepared from normal rat liver and from regenerating livers 4, 12, and 24 hr after hepatectomy by very gentle homogenization in medium A (ref 18) plus 5×10^{-3} *M* mercaptoethanol. After sedimentation of the mitochondria, 1 volume of 2.4% sodium deoxycholate was added to the supernatant, and the ribosomes were sedimented at 105,000 × *g* for 90 min. The ribosomal pellet was carefully resuspended in medium A without sucrose, layered on the top of a 10–34% sucrose gradient in Tris-succinate buffer pH 7.6 plus 5×10^{-3} *M* magnesium acetate, and centrifuged in the SW 99 rotor at 34,000 rpm for 75 min. Thirty fractions of 3 drops each were collected, and the optical densities at 260 mμ were measured.

Preliminary results show no appreciable differences between the pattern of distribution in sucrose gradient of the ribosomes from normal and from regenerating rat liver at the investigated times. This suggests that the differences in protein synthetic activity are not due to differences in the state of aggregation of the ribosomal population.

It is of interest to note that at 4 hr after hepatectomy, when the endoplasmic reticulum is known to undergo a marked reduction, a normal amount of polysomes is still present. This demonstrates the independence of the polysomal structure from the endoplasmic reticulum, even in cells where normally this membrane system is highly represented.

[16]T. Hultin and Alexandra Von Der Decken, *Exptl. Cell Res.* **13**, 83 (1957).

[17]F. O. Wettstein, T. Staehelin, and H. Noll, *Nature* **197**, 430 (1963).

[18]C. L. Robinson and G. D. Novelli, *Arch. Biochem. Biophys.* **96**, 459 (1962).

SPECIES DIFFERENCE OF S-RNA SHOWN BY DIFFERENT SUSCEPTIBILITY OF ITS AMINO ACID ACCEPTOR ACTIVITY TO *BACILLUS SUBTILIS* RNase

Susumu Nishimura G. D. Novelli

Introduction. – Species differences between S-RNA's from various sources identified by the ability to form different amino acyl–S-RNA's by a heterogeneous amino acid activating enzyme has been reported by several workers. It was also reported that differences between yeast and rat liver S-RNA's were shown by the countercurrent distribution technique.

This report also deals with species differences between yeast and rat liver S-RNA's found from the different sensitivities of each amino acid acceptor activity to *B. subtilis* RNase. We previously reported that acceptor activities of some amino acid specific S-RNA's were resistant to *B. subtilis* RNase, whereas other amino acid specific S-RNA's readily lost their acceptor activities when the bulk of S-RNA from *Escherchia coli* was incubated with *B. subtilis* in the presence of 0.01–0.001 *M* magnesium ion.[19] This finding led us to examine species differences of S-RNA's from various origins by using this technique.

Results and Discussion. – The acceptor activities of S-RNA for several amino acids were measured after hydrolysis of S-RNA by *B. subtilis* RNase. With respect to the pattern of sensitivity of acceptor activity for each amino acid, rat liver S-RNA was different from *E. coli* or yeast S-RNA, whereas *E. coli* S-RNA and yeast S-RNA were similar to each other. Rat liver S-RNA lost its lysine acceptor activity much more easily than *E. coli* or yeast S-RNA. On the contrary, glutamic acid and threonine acceptor activity of rat liver S-RNA was much more resistant to the RNase than *E. coli* or yeast S-RNA.

It is interesting to note that species differences shown by *B. subtilis* RNase treatment do not correspond to the differences detected by amino acid activating enzyme[20] or countercurrent distribution behavior.[21] For example, *E. coli* S-RNA

[19]Susumu Nishimura and G. D. Novelli, *Biochem. Biophys. Res. Communs.* **11**, 161 (1963).

[20]S. Benzer and B. Weisblum, *Proc. Natl. Acad. Sci. U.S.* **47**, 1141 (1961).

[21]J. Apgar and R. W. Holley, *Biochem. Biophys. Res. Communs.* **8**, 391 (1962).

and yeast S-RNA are very similar with respect to the RNase sensitivity of their amino acid acceptor activity with the nine amino acids (alanine, arginine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, and valine) thus far tested, whereas the activating enzymes can distinguish between *E. coli* and yeast S-RNA with respect to arginine, leucine, and lysine. *B. subtilis* RNase more readily inactivates the acceptor activity for threonine of yeast S-RNA than that of rat liver, whereas threonine S-RNA's from rat liver and yeast give the same position in countercurrent distribution. On the other hand, *B. subtilis* RNase treatment does not distinguish alanine S-RNA between yeast and rat liver, whereas alanine S-RNA from yeast and rat liver differ in countercurrent distribution behavior.

The different sensitivities of amino acid acceptor activity of S-RNA to the RNase was only observed when S-RNA was incubated with the RNase in the presence of Mg^{2+}. Formation of rigid secondary structure of S-RNA by interacting S-RNA with Mg^{2+} might contribute to the resistance of amino acid acceptor activity to the RNase. Therefore, such a species difference observed by RNase treatment may be due to the difference in magnitude of secondary structure of each species of S-RNA.

It is rather surprising that *B. subtilis* RNase cannot recognize species differences between *E. coli* and yeast S-RNA's. These results suggest universal structures in S-RNA molecules of both species responsible for amino acid acceptor activity.

TRANSFER RNA: REACTIVITY WITH AMINO ACID ACTIVATING ENZYMES FROM DIFFERENT ORGANISMS

K. B. Jacobson Susumu Nishimura
W. E. Barnett[22] R. J. Mans
Piero Cammarano

Introduction. – Transfer RNA is a collection of a variety of RNA molecules wherein one or more structurally specific RNA's exist for each amino acid. Specificity for the amino acid appears to result from a specific interaction of the RNA with the amino acid activating enzymes. Thus the enzyme, with ATP, produces a ternary complex: enzyme–amino acid–AMP. This complex then reacts with an RNA molecule specific for the amino acid to form S-RNA–amino acid. The chromatographic behavior of *Escherichia coli* S-RNA specific for several amino acids was described previously.[23] In this study, *E. coli* S-RNA was tested with different amino acid activating enzymes to determine whether the enzymes from different organisms and the *E. coli* enzyme transferred the amino acid to the same RNA molecule.

Results and Discussion. – The enzyme preparation for acylating S-RNA was prepared from *E. coli*, *Neurospora crassa*, maize seedlings, and mouse liver, as described previously.[24–27] The acylating of S-RNA was performed under conditions such that the S-RNA was rate-limiting.

With regard to the extent of amino acid acylation of the *E. coli* S-RNA, each enzyme preparation showed a different pattern. The enzyme from *E. coli* transferred leucine and valine to the greatest extent; the enzyme from *Neurospora* transferred leucine and phenylalanine best; the enzyme from maize was best with arginine; and the enzyme from mouse was equally efficient with leucine, valine, alanine, lysine, isoleucine, and serine. Eleven amino acids were examined for each enzyme. The pattern of amino acid acylation was different again when the homologous S-RNA for each enzyme was employed. A chromatogram of each of these amino acyl–RNA's was made by the DEAE-cellulose paper method[23] to determine its relative mobility.

The migration rate of leucyl-RNA was the same whether this amino acyl–RNA had been produced by *E. coli* enzyme or any of the other three; the same was true for valine, alanine, and isoleucine. Such a comparison was not possible for glycine, glutamic acid, and aspartic acid since none of the three heterologous enzymes gave significant activity. Three differences have been observed: the *Neurospora* enzyme transferred phenylalanine

[22]Fungal Genetics Group.

[23]K. B. Jacobson and Susumu Nishimura, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 139.

[24]Susumu Nishimura and G. D. Novelli, *ibid.*, p 132.

[25]W. E. Barnett and C. J. Wust, *ibid.*, p 139.

[26]R. J. Mans, *ibid.*, p 136.

[27]Piero Cammarano and G. D. Novelli, *ibid.*, p 135.

(1) and lysine (2) to *E. coli* S-RNA's that migrate quite differently from those produced by the *E. coli* enzyme; similarly for the maize enzyme, arginine (3) seems to be on a different RNA. These observations are being checked by another chromatographic procedure.

The most direct interpretation of these results will be in terms of substrate specificity wherein the structural features of the RNA molecule are compared and related to their affinity for the enzyme. A further interpretation could be attempted by considering the significance of these findings in relation to the RNA code for protein synthesis.

STUDIES ON ANTIBODY SYNTHESIS

C. J. Wust Clara L. Gall

Introduction. – Studies on antibody synthesis have continued both *in vivo* in the rat and in the cell-free incorporating system previously reported.[28] *In vivo* experiments are concerned with RNA metabolism following the antigenic stimulus.

Results and Discussion. – It was found that β-galactosidase, 60% purified, when given to rats in Freund's adjuvant, will elicit the production of a low titer of precipitating antibodies within 10 days after a single injection. A second injection, given 14 to 17 days after the first dose, will elicit a high titer of precipitating antibody within 7 days. The enzymic activity is not neutralized but is totally present in the precipitate. In the initial experiments designed to test the ability of the cell-free incorporating system to synthesize antibody, rats in the secondary response were killed at 7 days when a high titer of antibody was found in the serum. Spleen ribosomes were prepared as previously described[28] and were incubated with optimal concentrations of liver S-RNA and enzyme preparations obtained from nonimmunized rats. There was a significant increase in the ability of the ribosomes to bind enzyme activity after incubation. Ribosomes alone bound some enzyme activity, and this finding confirms the report (dealing with another enzyme) that ribonucleoprotein-bound antibody is present and could be demonstrated with the enzyme D-glyceraldehyde-3-phosphate dehydrogenase.[29] The increase in binding ability after incubation was dependent on the use of immune ribosomes, energy, and liver enzymes and was further increased with the addition of S-RNA. Although the increase in binding was significant, the system is being examined to obtain higher levels of synthesis. One area of study is the age of the ribosomes during the immune response. Ribosomes in the early experiments were obtained when the titer of circulating antibody was maximal in the serum. This indicates that the *in vivo* synthesis may have been declining since the presence of circulating antibody is a release phenomenon from cell to serum. Ribosomes will be obtained before circulating antibody is maximal.

After the antigenic stimulus is administered, RNA metabolism increases prior to the appearance of circulating antibody.[30] The RNA is being isolated at that time to determine if it contains information about antibody specificity. This information will be tested both *in vivo* and in the cell-free system.

Another aspect of RNA metabolism has been the use of the drug actinomycin D following the antigenic stimulus. This drug presumably affects only the DNA-dependent RNA polymerase. If the drug depresses the synthesis of circulating antibody, then the normal sequence of protein synthesis involving DNA should be realized, although the information for specificity may reside in a later step where antigenic fragments would contribute. The use of the drug following the second injection of β-galactosidase has been studied. If the drug is administered 3 to 24 hr after the antigen, there is a depression in the titer of circulating antibody observed at nine days. There is no significant depression if the drug is given at 72 hr. Sheep erythrocytes also have been used as antigen since a single intraperitoneal injection of 1 ml of a 5% suspension elicits maximally high titers within five days. Actinomycin D given 3 hr or more after this antigen is administered has no effect on antibody titer determined at six days. It is possible that the RNA metabolism with this antigen would be affected earlier than at 3 hr. Experiments are now in progress to determine if the simultaneous administration of drug with sheep

[28] C. J. Wust, *ibid.*, p 138.

[29] C. J. Wust and G. D. Novelli, *J. Immunol.* **93**, 734 (1963).

[30] C. J. Wust and L. Astrachan, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1962*, ORNL-3267, pp 129–31.

erythrocytes will alter the titer and time course of appearance of circulating antibody.

PURIFICATION AND CHARACTERIZATION OF THE pH 7.5 ESTERASE FROM MAIZE

Katherine H. McGrath G. D. Novelli

In a previous report[31] we mentioned that the pH 7.5 esterase could be separated from the other esterases of maize by a gradient elution from a cellulose acetate column. Although this procedure gave an enzyme that was 30- to 50-fold purified, quantitation of the operation indicated that the yield of enzyme was very low. Since that time we have found that putting the dialyzed ammonium sulfate fraction over a DEAE column that does not absorb the esterase yields a product that is nine to ten times purified, with a recovery of 75%. Preliminary studies with a carboxymethylcellulose column suggest that most of the maize esterases are not retained by the column, whereas the pH 7.5 esterase is held up and can be eluted with 0.01 *M* citrate buffer at pH 6.4. The quantitative aspects of this step have not yet been assessed.

We have found that seedlings germinated in the corn pilot plant give the best yield of esterase if germination is allowed to proceed for five days instead of three days, as with tray germination. If the present purification procedure has successfully resolved the pH 7.5 esterase from the other maize esterases, we should be able to use a quantitative colorimetric assay rather than gel electrophoresis. This would allow for a much faster purification procedure, which could be used on the scale necessary for our purposes.

FURTHER STUDIES ON THE AMINO ACID INCORPORATING SYSTEM ISOLATED FROM MAIZE SEEDLINGS

R. J. Mans

Introduction. – Studies on the biosynthesis of protein in higher plants are being continued with the amino acid incorporating system isolated from maize seedlings.[32] This system incorporates any of several amino acids into ribosomal protein in the presence of energy, several cofactors, and a 105,000 × *g* supernatant component. Although protein synthesis should require the simultaneous presence of all 20 amino acids, incorporation of a single amino acid by this system is inhibited by the addition of the other 19 amino acids. The nature of this inhibition and attempts to overcome it is the subject of this report.

Results and Discussion. – Seedlings, upon germination, mobilize much of their storage proteins by degrading the protein to free amino acids[33] that are released to the supernatant component. It is postulated that the high level of endogenous amino acids masks the requirement for added amino acids in the cell-free system. Attempts to remove the endogenous amino acids by isoelectric precipitation and overnight dialysis of the supernatant result in the inactivation of the soluble enzymes required for incorporation.

Treatment of a stabilized supernatant component[34] by gel filtration (i.e., passage over Sephadex G-25) removes the endogenous amino acids from the supernatant, with retention of the incorporating activity. By equilibration and elution of the Sephadex column with the same medium used in preparation of the supernatant component (0.45 *M* sucrose, 0.05 *M* Tris pH 7.6, 0.0005 *M* $MgCl_2$), all the supernatant activity is recovered from the column. Furthermore, the leucine incorporation catalyzed by the treated supernatant is doubled by addition of an equimolar mixture of the other 19 amino acids.

It has not been possible to demonstrate an absolute requirement for added amino acids as has been reported for the incorporating system isolated from peas.[35] Preliminary experiments suggest that free amino acids arise during incubation by autolysis of either the supernatant or particle protein components, or both, and may account for the failure to demonstrate an absolute requirement for amino acids. This aspect of the problem is currently under investigation.

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[31] Katherine H. McGrath and G. D. Novelli, *ibid.*, p 134.

[32] R. J. Mans and G. D. Novelli, *Biochim. Biophys. Acta* **50**, 287 (1961).

[33] J. M. Lawrence, K. M. Day, and J. E. Stephenson, *Plant Physiol.* **34**, 668 (1959).

[34] R. J. Mans and G. D. Novelli, in press.

[35] G. D. Webster, *Arch. Biochem. Biophys.* **85**, 159 (1959).

INCREASES IN ENDOGENOUS INHIBITORS OF MAIZE AMINO ACID INCORPORATING SYSTEM DURING GERMINATION

Leon S. Dure III R. J. Mans

Introduction. – In studying protein synthesis in developing maize seedlings, it has been observed previously[36] that the ability of cell-free preparations from maize shoots to incorporate amino acids into acid-insoluble protein decreases markedly as the seedling matures. This study was initiated to determine the factor(s) responsible for the decrease in incorporating activity. The amino acid incorporating system from maize shoots has been refined[37] since this initial observation and, therefore, it was necessary to reestablish the *in vitro* loss of amino acid incorporating activity with increasing age after germination.

Results and Discussion. – Active components of the incorporating system (i.e., ribosomes and 105,000 × *g* supernatant) were prepared from etiolated maize seedlings at 2, 3, 5, 7, and 9 days after germination. The components were recombined, and the amino acid incorporating activity was determined. Activity measurements obtained by crossing the older components with those of 2-day-old seedlings were also made. The loss of amino acid incorporating activity with increasing age after germination was found to occur with the more refined system. Further, mixing of either the supernatant or ribosomal components of the older seedlings with those from 2-day-old seedlings depressed incorporation over that obtained with the 2-day-old materials alone. The loss in activity of older seedling components is associated with both the supernatant and the ribosomal components.

Two factors known to be present in the supernatant could account for the loss in activity with maturation and the inhibition of the more active 2-day-old system. These are the presence of free amino acids[36] and ribonuclease.[37] A large increase in the amino acid pool arising from autolysis of storage protein during maturation of the seedling would dilute the isotopic amino acid added to measure incorporation. The dilution of the added isotope would depress the amount of incorporation detected and thus give rise to an apparent decrease in activity. Endogenous RNase is a potent inhibitor of the maize incorporating system, and an increase in extractable levels of RNase with seedling maturation could account for the dramatic decrease observed in incorporating activity.

Both these factors were investigated. The free amino acid pool was found to increase in supernatants prepared from older seedlings. However, the maximum increase observed (in 7-day-old seedlings) was only twice that present in 2-day-old supernatant and not sufficient to account for the 20-fold decrease in incorporation. On the other hand, RNase which was not detectable in 2-day-old supernatants was detected in 3-day-old supernatants and increased to very high levels throughout 7 days of germination.

It seems that the rapid decrease in incorporation and the inhibitory nature of supernatant components from older seedlings is due to the presence of an increasing level of RNase in maturing seedlings. Preliminary studies indicate that some of the loss of activity of the ribosomal component from older seedlings is also due to the destruction of essential ribonucleic acids by endogenous RNase. Further investigation of protein synthesis during maize seedling development is directed toward overcoming the inhibition of incorporation by endogenous RNase. Bentonite and dextran sulfate, efficient absorbers of basic proteins such as RNase, are being included in homogenates from which the supernatant and ribosomal components are prepared in an effort to remove endogenous RNase.

[36] R. J. Mans and G. D. Novelli, *Biochim. Biophys. Acta* **50**, 287 (1961).

[37] R. J. Mans and G. D. Novelli, in press.

STUDIES ON THE EFFECTS OF LIGHT ON PROTEIN SYNTHESIS IN PLANTS

G. R. Williams R. J. Mans
G. D. Novelli

Introduction. – Etiolated plants are characterized by (1) the absence of chlorophyll, (2) relatively low levels of protein and lipid material, and (3) the presence of smaller plastids which lack normal ultrastructure. Upon exposure to light, a rapid increase in RNA followed by enhanced protein synthesis has been demonstrated

in *Euglena gracilis.*[38] Similarly, the levels of protein and lipid in plastids from etiolated bean leaves increase twofold after a brief period of illumination with red or white light.[39] TPNH-specific triose phosphate dehydrogenase activity in red kidney bean leaves can be demonstrated only by a brief exposure to red light; further, this effect is reversed by a subsequent treatment with far-red light.[40] The level of nitrate reductase activity in cell-free extracts of corn shoots increases twofold within 5 hr after exposure to light.[41] Thus, some portion of the light-induced increase in protein level may represent the synthesis of specific enzymes absent or present only in lower concentrations in etiolated leaves.

The present investigation was undertaken to study the effects of light on protein synthesis in cell-free preparations from plant material in an effort to clarify the intermediate steps and the mechanism of control involved.

Results and Discussion. – We have examined the amino acid incorporating activity of particles prepared from two- and three-day-old etiolated maize seedlings[42] after brief illumination. Upon exposing two-day-old seedlings to white light, the incorporating activity was enhanced 15 to 53%. The activity of particle preparations from illuminated three-day-old shoots was further enhanced, yielding as much as a twofold stimulation, although the overall activity of these older systems was much lower. The effect of light upon the rate of amino acid incorporation is associated exclusively with the particle fraction.

Particles from etiolated shoots have been examined using sucrose gradient centrifugation techniques. Although the monomeric unit (about 70 S) appeared to predominate in our preparations, a series of polymers ranging from 100 to 200 S were observed. When particle preparations were incubated briefly in a reaction mixture and subsequently separated on sucrose gradients, the specific activity of the polymers was much greater than the 70 S material.

A cursory examination of three-day preparations revealed a lower level of heavy particles, which is consistent with the general depression of incorporating activity in these preparations from older seedlings. Preliminary results indicate that the shoots exposed to light yield an increased level of heavy particles which could account for the observation that particles from treated shoots are more active.

Further studies concerning the effect of light on the level of polysomes together with possible effect of light on the synthesis of messenger RNA are currently being pursued. In addition, the action spectrum of the light effect upon amino acid incorporation by cell-free preparations will be undertaken in order to correlate the results of this investigation with the developmental phenomena observed in intact plants.

CELL-FREE AMINO ACID INCORPORATING SYSTEM FROM MAIZE TISSUE CULTURE

J. E. Graebe G. D. Novelli

Introduction. – Earlier reported studies[43] on a cell-free amino acid incorporating system from large-scale tissue culture of maize endosperm have been continued. The growth technique has been modified to yield a constant supply of tissue with a minimum of maintenance work. Efforts have been made to find a practical method for preparing ribosomes from large batches of tissue to increase the activity of the obtained particles and to find the reason why the high-speed supernatant fraction of the system does not support incorporation.

Results and Discussion. – The tissue is maintained submerged in a synthetic medium in two aerated 6-liter flasks. Approximately every ten days one culture is harvested, yielding 350–500 g of tissue, fresh weight. The other culture is divided into two flasks, and incubation is continued after addition of 3 liters of fresh medium to each.

The tissue has been homogenized either by batchwise grinding in a ground-glass homogenizer or by passing it through a Gaulin laboratory homogenizer. The glass homogenizer produces

[38] G. Brawerman *et al.*, *Biochim. Biophys. Acta* **55**, 329 (1962).

[39] J. L. Mego and A. T. Jagendorf, *Biochim. Biophys. Acta* **53**, 237 (1961).

[40] A. Marcus, *Plant Physiol.* **35**, 126 (1960).

[41] R. H. Hageman and D. Flesher, *Plant Physiol.* **35**, 700 (1960).

[42] R. J. Mans and G. D. Novelli, *Biochim. Biophys. Acta* **50**, 287 (1961).

[43] J. E. Graebe and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 137.

a somewhat higher final yield of particles, but the method is slow and tedious, requiring 3 hr for 1 lb of tissue. The same amount is homogenized in 2 min by the Gaulin homogenizer. The resulting preparations have a specific activity as high as or higher than that of the preparations made in the glass homogenizer, but the yield of particles is, as yet, impractically small. Thus when "ergosomes" are made according to the method by Wettstein *et al.*,[44] the glass homogenizer has yielded up to 8 mg of particle protein per 200 g of tissue, whereas no more than 0.1–0.3 mg has been obtained with the Gaulin homogenizer.

Attempts have been made to decrease the time of preparation of the particulate fraction by concentration of the postmitochondrial supernatant with dry, coarse-grade Sephadex G-25 (ref 45). By this method, 1480 ml of postmitochondrial supernatant was reduced to 162 ml in two batches within 1 hr, supposedly concentrating proteins and particles without affecting the concentration of smaller ions. This treatment seems to have no adverse effect on the specific activity of the final ribosomal preparation. Its effect on the yield has not been ascertained.

It is common practice to clarify the final suspension of ribosomes by a low-speed centrifugation (20,000 × *g*) and discard the pellet. In the present system the pellet thus obtained contains a considerable amount of material, often with a specific activity as high as that of the particles remaining in suspension.

For the incorporation of leucine-C^{14} into protein, the system typically requires the presence of a small amount of the 105,000 × *g* soluble fraction in addition to the particulate fraction. This soluble fraction of the tissue culture system is inactive. The ribosomal activity therefore has to be assayed by cross reaction with the soluble fraction from maize seedlings. Such a mixed system is inhibited by the addition of a small amount of the soluble fraction from the tissue culture preparation both with respect to incorporation and to charging. The measured RNase activity of the fraction is not enough to account for the potent inhibition. The inhibition persists without loss of activity after 5 min of vigorous boiling. If just enough of the inhibiting fraction is added to the active system to cause 75% inhibition, the inhibition is the same whether the inhibitor is added at the start of the incorporation experiment or preincubated separately with the active fractions 5 min before. These results make an enzymatic breakdown of the active components an unlikely reason for the inhibition. More likely the supernatant fraction from the tissue culture contains ions of heavy metals, originally accumulated in the large vacuoles of the cells. The inhibition is not removed by plain dialysis against buffer, but it is somewhat decreased by passing the solution over a Sephadex G-25 column, recovering the fractions with large molecules or by treatment with a cation exchanger. Since the inhibitor is recovered with the large molecules after gel filtration, it may consist of heavy ions bound to protein, in which case it might be removed by a combination of dialysis with Versene and gel filtration.

Incorporation as high as 70 micromicromoles of leucine-C^{14} per mg of particle protein during 30 min has been obtained with the mixed system of tissue culture particles and seedling supernatant (an earlier reported[43] figure of 100 micromicromoles/mg should be corrected to 10). The system, therefore, is potent enough to offer attractive possibilities for further studies, particularly if the yield of particles from the tissue can be increased.

SURFACE MONOLAYER STUDIES

A. P. Pfuderer

Introduction. – Preliminary surface studies have been started on biological polymers. To this end a horizontal float-type surface balance has been built and is currently being modified to permit simultaneous vertical Wilhemy plate measurements. When completed, the horizontal balance will be used to record isotherms over the entire pressure range, and the vertical balance will be used for the "gaseous" region.

Results and Discussion. – The film pressure vs surface area isotherms of β-lactoglobulin, serum albumin, and poly-L-phenylalanine were observed at low surface pressures. Two different

[44]F. O. Wettstein, T. Staehelin, and H. Noll, *Nature* **197**, 430 (1963).

[45]P. Flodin, B. Gelotte, and J. Porath, *Nature* **188**, 493 (1960).

methods of treating the data were examined, the FA vs F plot of Bull[46] and the new equation of state proposed by Fowkes.[47]

The method of Trurnit[48] was used to deposit the films on the surface using a 2-cm glass rod. This resulted in complete spreading for serum albumin and poly-L-phenylalanine, but only about 70% for β-lactoglobulin, as judged by the specific area of the deposited film.

The surface molecular weight of β-lactoglobulin when corrected for incomplete spreading was within 10% of the literature value of 18,000 for the monomer, while the serum albumin results were within 5% of the literature value of 65,000.

The three phenylalanine samples had molecular weights of 4000, 13,000, and 63,000, respectively, as computed from sedimentation rate and intrinsic viscosity data. The surface molecular weights of these three samples on water substrates were of the order of one million, showing a surface aggregate in agreement with Mishuck and Eirich,[49] while the molecular weights on concentrated aqueous solutions of urea or dimethylformamide were of the order of 30,000–50,000 for all three samples. This phenomenon was also reported by Mishuck and Eirich, who had no estimate of the molecular weight of their samples and believed they were obtaining a true molecular weight. It seems possible that the urea and dimethylformamide solutions have given the polyphenylalanine aggregate increased flexibility, resulting in a lower apparent molecular weight[50] without actually rendering them monodisperse.

The Fowkes equation of state proved to be a precise way of treating high-molecular-weight samples. There seems to be no reason why the Fowkes method of plotting film molecular weights should not be applicable to very large molecules, the only limitation being the cleanliness of the surface. As the molecule studied increases in size, there are fewer molecules present per unit weight and contamination of the surface by small molecules becomes increasingly critical.

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[46] H. B. Bull, *J. Biol. Chem.* **185**, 27 (1950).

[47] F. M. Fowkes, *J. Phys. Chem.* **66**, 385 (1962).

[48] H. J. Trurnit, *J. Colloid Sci.* **15**, 1 (1960).

[49] E. Mishuck and F. R. Eirich, *J. Polymer Sci.* **16**, 397 (1955).

[50] J. T. Davies, *Biochim. Biophys. Acta* **11**, 165 (1953).

Hormonal Control

Two systems are being investigated in which steroid hormones play a regulatory role in protein biosynthesis. The first of these has been intensively studied in this laboratory and involves induction of hepatic enzymes *in vivo* by administration of adrenocorticosteroids. The current effort with this system is devoted to a study of the nucleic acid components that may direct specificity in protein synthesis. The effect of the steroid hormones on such nucleic acid metabolism is detailed below. The other system involves the use of estrogenic hormones to stimulate a rapid increase in the rate of protein synthesis in the uterus of ovariectomized rats. The effort with this system has been to develop a stable cell-free amino acid incorporating system to enable a study of the *in vitro* effect of estrogenic hormones on this system.

HORMONAL CONTROL OF HEPATIC ENZYME SYNTHESIS

F. T. Kenney F. J. Kull
W. L. Albritton

Introduction. – Our study of the mechanism whereby adrenal hormones accelerate the synthesis of certain hepatic enzymes has recently been concerned with the nucleic acid components thought to direct specificity in protein synthesis.

Results and Discussion. – Adrenalectomized rats were treated with hydrocortisone for varying periods of time and given a pulse of P^{32} (1 mc) for 20 min before they were killed. Livers were fractionated into nuclear and cytoplasmic fractions, and then RNA was purified from each of these by hot salt extraction and removal of contaminants on DEAE paper. The specific radioactivity of RNA labeled in this fashion was taken as a measure of the rate of *in vivo* RNA synthesis, after correction for variation of precursor pools. These experiments revealed the following: (1) the rate of *cytoplasmic* RNA synthesis is not influenced by hormone treatment; (2) the rate of *nuclear* RNA synthesis is increased after a lag of about 30 min, increasing to three- to fivefold after 4 hr; and (3) accumulation of the induced tryosine transaminase begins shortly after increased nuclear RNA synthesis is apparent, a

result consistent with the interpretation that enzyme induction is secondary to a primary hormonal effect on the synthesis of nuclear RNA.

Characterization of the RNA induced by hydrocortisone was undertaken in an attempt to delineate its role in enzyme synthesis. The pattern of distribution of pulse-labeled RNA in sucrose gradients was found to be unchanged by induction; in both induced and noninduced livers this RNA was found to be extensively heterogeneous, ranging in size from 40 to 8 S. The possibility that this represents degraded fragments of a still larger RNA is now being studied. Base composition analysis of the newly labeled RNA from noninduced livers indicated a makeup like that of the total nuclear RNA, determined chemically. This composition was not appreciably altered by hormone treatment. Assay for messenger RNA activity – defined here as capacity to stimulate amino acid incorporation in a fully supplemented *in vitro* system prepared from rat liver – indicated that there was little or no change in this parameter on induction. These results suggest that the stimulation of nuclear RNA synthesis is a general one (i.e., that the hormone-treated liver is simply synthesizing more rapidly the same kind of RNA that is synthesized in the absence of adrenal hormones). In functional terms, this probably involves messenger RNA, transfer RNA, and perhaps a precursor form of ribosomal RNA. That the hormone increases the synthesis of all these may indicate that selective induction of particular enzymes is more apparent than real.

Experiments as yet incomplete indicate an increase in the activity of the DNA-requiring RNA polymerase of liver nuclei, following hydrocortisone treatment. This is presumably the basis for increased RNA synthesis and may reflect the immediate site of hormonal action in enzyme induction. It is not yet known whether increased activity of this enzyme reflects *de novo* synthesis or some activation mechanism. The enzyme as assayed is a crude DNA-protein complex, and it is recognized that many factors other than an actual increase in enzyme protein could result in an increased activity in *in vitro* assays. This aspect is now being investigated.

[51] D. L. Greenman and F. T. Kenney, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963,* ORNL-3427, p 141.

EFFECT OF ESTROGEN ON PROTEIN SYNTHESIS IN THE RAT UTERUS

D. L. Greenman F. T. Kenney

Introduction. – It is well established that estrogenic hormones cause a rapid increase in the rate of protein synthesis in the uterus of the ovariectomized rat. The cell-free amino acid incorporating system which we have previously described[51] has been used to study the manner in which estrogen stimulates this synthetic process.

Results and Discussion. – Ribosomal preparations were obtained from rats subjected to various experimental manipulations. We have examined these preparations with respect to their amino acid incorporating ability and their pattern of sedimentation in a sucrose density gradient.

When we assayed these ribosomes in the presence of the uterine supernatant fraction, their apparent amino acid incorporating ability dropped 30% within a 24-hr period following ovariectomy. However, ribosomes obtained from rats ovariectomized for two, four, or seven days exhibited activity equal to or greater than that obtained from ribosomes of intact rats. Administration of a single dose of estradiol (10 μg) 3 to 5 hr before killing ovariectomized rats increased ribosomal activity as much as 70%.

Preliminary results suggest that the assay of ribosomes in the presence of liver supernatant fluid alters the pattern of ribosomal activity which we observed when the uterine supernatant fraction was used. Thus, using the soluble fraction from liver, a progressive drop in apparent ribosomal activity is seen to occur as the postcastration period is increased. We have also shown that the addition of S-RNA stimulates amino acid incorporation only in the presence of the uterine supernatant fluid. Therefore, it appears that the uterine supernatant fraction may be limited by a low concentration of transfer RNA. Thus, apparent ribosomal activity obtained using the uterine fraction may be a function of the amount of S-RNA bound to the ribosomes at the time of isolation. This possibility is being investigated.

Analysis of ribosomes by density gradient centrifugation has generally shown a dominant optical density peak in the region corresponding to 100 S, with a somewhat smaller peak sedimenting at about 70 S. More material sedimenting faster than

the 100 S peak appears to be present in ribosomes obtained from rats given a single injection of estradiol as compared to ovariectomized controls. Following repeated injections of estradiol over a 48-hr period, a striking rise in the 70 S component has been observed.

Mammalian Cell Regulation

The reports on mammalian cell regulation constitute a continuing study of the lactic dehydrogenase isozymes. We are attempting to understand the reason why some of the isozymes break up into subbands, as part of a study of the control mechanism responsible for the different distribution of the five major isozymes in different tissues during development.

STUDIES ON LACTIC DEHYDROGENASE ISOZYMES

P. J. Fritz K. B. Jacobson

Introduction. – The five major isozymes of lactic dehydrogenase (LDH) have been resolved by electrophoresis on polyacrylamide gels into multiple subbands as previously described.[52] A theory to account for these subbands involving differential coenzyme binding was advanced. In an attempt to confirm the theory, a substance was extracted from commercial crystalline rabbit muscle LDH which has spectral and chromatographic properties similar to the carbonyl addition products of nicotine adenine dinucleotide (NAD), as described by Dolin and Jacobson.[53]

Results and Discussion. – Commercial crystalline rabbit muscle LDH was boiled in potassium carbonate buffer pH 10.0 and dialyzed against water overnight. Concentration of the dialysate and examination of its properties revealed the presence of a fluorescent substance which had absorption peaks at 250 and 360 mμ at neutral and acid pH and a peak at 400 mμ in alkaline pH. These properties and its chromatographic behavior made it look very much like a carbonyl addition compound to NAD, as described by Dolin and Jacobson.[53]

It seemed reasonable that if such a compound were indeed occurring in nature, it might be the pyruvate addition compound since pyruvate is a substrate for LDH. However, synthesis of authentic pyruvate-NAD and comparison of its properties to the naturally occurring compound revealed fundamental differences.

Since acetaldehyde could arise by decarboxylation of pyruvic acid, the acetaldehyde-NAD addition compound was prepared. The properties of this compound and the naturally occurring one have many similarities; however, the identity of the two has not been established with certainty.

The naturally occurring compound inhibits the activity of LDH as determined in the spectrophotometric assay. This observation has led to speculation concerning the biological function of the compound, especially its possible role in metabolic control mechanisms. It has not yet been determined whether the presence or absence of this compound on the enzyme has any effect on the multiple electrophoretic bands.

CHANGES IN THE ISOZYMES OF LACTIC DEHYDROGENASE IN MICE WITH MUSCULAR DYSTROPHY

K. B. Jacobson J. B. Murphy

Introduction. – Lactic dehydrogenase occurs in many animals as five or more proteins as distinguished by gel electrophoresis. It has been proposed[54,55] that the control of the five forms is due to two genes in addition to some unknown organ specific mechanism. Neither form of control has been demonstrated experimentally. Wieme and Herpal[56] reported that the muscular dystrophy of humans was accompanied by a marked alteration in the LDH isozyme distribution; Kaplan and Cahn[57] observed similar events in chickens with

[52] P. J. Fritz and K. B. Jacobson, *Science* **140**, 64 (1963).

[53] M. I. Dolin and K. B. Jacobson, *Biochem. Biophys. Res. Communs.* **11**, 102 (1963).

[54] E. Appella and C. C. Markert, *Biochem. Biophys. Res. Communs.* **6**, 171 (1961).

[55] R. D. Cahn, N. O. Kaplan, and E. Zwillig, *Science* **136**, 962 (1962).

[56] R. J. Wieme and J. E. Herpal, *Nature* **194**, 287 (1962).

[57] N. O. Kaplan and R. D. Cahn, *Proc. Natl. Acad. Sci. U.S.* **48**, 2123 (1962).

this malady but mentioned that a genetically controlled muscular dystrophy in mice did not show such changes. We have examined dystrophic mice to determine what changes in the LDH isozyme pattern occur.

Results and Discussion. – A genetically controlled muscular dystrophy occurs in 129 Re mice wherein the lower back and hind leg muscles are reduced in weight to 10–20% of normal and the hind legs do not function. The front legs remain functional although the amount of muscle is less than normal.

The LDH isozyme distribution is abnormal in the more seriously afflicted muscle but is nearly normal in the front leg muscle. The alteration that occurs in the isozyme distribution in the hind leg muscle is similar to that in the human and chicken muscle in that there is a marked decrease in the slowest isozyme band. Although the normal liver isozymes are distributed similarly to the muscle, no change in the liver isozymes was observed.

The plasma LDH of dystrophic mice is also abnormal in isozyme distribution. Since the mouse is not afflicted until approximately four weeks of age, the correlation between the plasma isozyme pattern and the onset of the dystrophy can be measured. These studies may aid in revealing the mechanism for the control of enzyme synthesis and particularly the control of isozyme distribution.

Energy Metabolism

The reports on energy metabolism describe continuing studies of the energetics of sulfur metabolism in autotrophic bacteria. Since these organisms derive most of their metabolic energy from reactions involving inorganic sulfur compounds, they provide excellent material for the study of factors and conditions that control the flow of metabolic energy into biosynthetic pathways.

COUPLING OF ENERGY-YIELDING REACTIONS WITH CARBON DIOXIDE FIXATION IN EXTRACTS OF *THIOBACILLUS THIOPARUS*

E. J. Johnson H. D. Peck, Jr.

Introduction. – A number of years ago Umbreit and his coworkers,[58] working with *T. thiooxidans*, proposed that energy production and CO_2 fixation were separate events in time and, consequently, that it was necessary to transfer energy, presumably in the form of adenosine triphosphate (ATP), between the site of energy formation and that of CO_2 fixation. Since that time, numerous investigators have attempted to confirm the observations of Umbreit; however, the results have been uniformly equivocal.[59]

Recently a mechanism for the formation of high-energy phosphate during the oxidation of sulfite to sulfate in extracts of *T. thioparus* has been described by workers at this laboratory.[60] It was proposed, therefore, to employ this energy-generating system to drive the energy-requiring CO_2-fixing system comprised of the enzymes phosphoribulokinase and carboxydismutase, in the presence of $C^{14}O_2$, ribose-5-phosphate (R-5-P), and Mg^{2+} and to demonstrate the nature of the components required for the coupling of these two enzyme systems.

Results and Discussion. – Cell-free extracts of *T. thioparus* that were capable of fixing $C^{14}O_2$ into the aqueous phase were prepared using the French pressure cell. This reaction has been shown to require ATP, R-5-P, and Mg^{2+} and is presumed to involve the enzymes, phosphopentokinase and ribulose-1,5-diphosphate carboxydismutase. The products of the reaction are being investigated.

In the absence of ATP, a stimulation of CO_2 fixation by sulfite oxidation has been demonstrated either when the $C^{14}O_2$ is added in air simultaneously with the sulfite or sequentially under helium. Although an absolute dependence upon adenosine monophosphate (AMP) has not been obtained, the system (CO_2 fixation) is stimulated by the addition of 1 to 3 micromoles

[58] K. G. Vogler and W. W. Umbreit, *J. Gen. Physiol.* **26**, 157 (1942).

[59] S. R. Elsden, in *The Bacteria*, vol III, p 5, Academic, New York, 1962.

[60] H. D. Peck, Jr., and E. Fisher, *J. Biol. Chem.* **237**, 190 (1962).

of AMP. Greater amounts of AMP completely inhibit this fixation of $C^{14}O_2$. It is anticipated that it will be possible to demonstrate sequentially the esterification of orthophosphate during sulfite oxidation and its utilization for the fixation of CO_2 in these extracts.

THE INHIBITION OF DISSIMILATORY SULFATE REDUCTION BY FLUOROPHOSPHATE

Sister M. Regina Lanigan H. D. Peck, Jr.

Introduction. – Whole cells of *Desulfovibrio desulfuricans* reduce sulfate to sulfide in the presence of molecular hydrogen, and the reduction of sulfate (but not sulfite or thiosulfate) is inhibited by group VI anions (SeO_4^{2-}, CrO_4^{2-}, MoO_4^{2-}, WO_4^{2-}),[61] 2,4-dinitrophenol,[62] and fluorophosphate.[63] The effect of group VI anions on sulfate reduction has been shown to be due to the specific inhibition of ATP-sulfurylase by these anions. The effect of DNP on the reduction of sulfate by whole cells of *D. desulfuricans* appears to be the result of the inhibition of the "anaerobic oxidative phosphorylation" occurring during, and required for, the reduction of sulfate with molecular hydrogen.

Since DNP is technically difficult to use as an inhibitor of oxidative phosphorylation in extracts of this organism, it was of interest to determine the mechanism by which fluorophosphate inhibits sulfate reduction.

Results and Discussion. – The reduction of sulfate to sulfide with H_2 in whole cells of *D. desulfuricans* is inhibited by fluorophosphate; however, the reduction of thiosulfate, which does not require ATP, is unaffected by this compound. In cell-free extracts, neither the reduction of sulfate nor thiosulfate is inhibited by fluorophosphate. These results indicated that fluorophosphate is not directly inhibiting the enzymes required for the reduction sulfate but is probably inhibiting either the entrance of sulfate into the cells or the generation of high-energy phosphate required for the reduction of sulfate. Since the reduction of sulfate was not inhibited by fluorophosphate when reducing power and ATP were supplied by pyruvic acid, it does not appear that this inhibitor is preventing the entrance of sulfate into the cells. Therefore, it was concluded that fluorophosphate is functioning in a manner similar to that of DNP by uncoupling the anaerobic oxidative phosphorylation occurring during the reduction of sulfate with H_2.

In cell-free extracts, fluorophosphate inhibits a hydrogen-stimulated exchange of orthophosphate into ATP, and it is hoped that fluorophosphate will be useful in defining this anaerobic oxidative phosphorylation in cell-free systems.

[61] H. D. Peck, Jr., *Proc. Natl. Acad. Sci. U.S.* **45**, 701 (1959).

[62] H. D. Peck, Jr., *J. Biol. Chem.* **235**, 2734 (1960).

[63] J. R. Postgate, *J. Gen. Microbiol.* **6**, 128 (1952).

THE OCCURRENCE OF ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE IN EXTRACTS OF *CHROMATIUM SP.*

H. D. Peck, Jr. J. T. Davidson

Introduction. – The occurrence of adenosine 5′-phosphosulfate (APS) reductase has been reported only in the "sulfate reducing bacteria" and in the *Thiobacilli*. In both groups of microorganisms, inorganic sulfur-containing compounds are involved in essential energy-yielding reactions. Since inorganic sulfur-containing compounds are also utilized by certain photosynthetic microorganisms during photosynthetic growth, it was of interest to determine whether APS reductase was present in extracts of *Chromatium* grown photosynthetically with reduced sulfur compounds.

Results and Discussion. – *Chromatium sp.* was grown photosynthetically on a completely inorganic medium containing carbonate, sulfide, thiosulfate, and mineral salts. Cell-free extracts were prepared in 0.5 *M* sucrose by sonic oscillation and, after removal of whole cells by low-speed centrifugation, the chromatophores were separated from the soluble proteins by high-speed centrifugation.

APS reductase activity is usually determined by spectrophotometrically following the reduction of $Fe(CN)_6^{3-}$ according to the reaction:

$$SO_3^{2-} + AMP + 2Fe(CN)_6^{3-} \longrightarrow APS + 2Fe(CN)_6^{4-}.$$

As the chromatophores are dark red, it was not possible to utilize the $Fe(CN)_6^{3-}$ assay with these extracts. Instead, S^{35}-labeled sulfite was

added to reaction mixtures, and the radioactive APS was isolated by paper electrophoresis.

APS reductase has been found to be located almost entirely on the chromatophores. The formation of APS requires the presence of AMP and $Fe(CN)_6^{3-}$ and is not stimulated by light. The nucleotide specificity of this enzyme has been investigated, and dAMP was found to be more active in the system than AMP. The specific activity of APS reductase, 1 micromole of APS per milligram of protein per hour, suggests that the enzyme is involved in the sulfur metabolism of *Chromatium*; however, more experimentation will be required to establish this point.

SEDIMENTATION STUDIES ON ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE

H. D. Peck, Jr. Daniel Holladay
A. P. Pfuderer

Introduction. – Adenosine 5′-phosphosulfate reductase (APS reductase) preparations in solution normally exhibit small amounts of a higher molecular weight species. As a preliminary step to molecular weight studies on the enzyme, it was decided to investigate this aggregation, as well as the fragmentation of the enzyme with *p*-hydroxy-mercuribenzoate (*p*-CMB).[64]

[64]H. D. Peck, Jr., and T. E. Deacon, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p 167.

Results and Discussion. – The main component of APS-reductase was found to have an $s_{20,w}$ of 10.5 Svedberg units. A diffusion constant of the main component alone was obtained in the ultracentrifuge by a synthetic boundary experiment at low speed. The diffusion coefficient of the trailing half of the schlieren peak which would be almost pure in the main component was $D_{20,w} = 6.9 \times 10^{-7}$ cm^2/sec. This would lead to a molecular weight of 150,000 for this component. This method would be the only way to determine the molecular weight of the main component without actually isolating it.

The average molecular weight of the mixture was found to be 300,000 for a preparation which had approximately 20–30% of the heavy component. The sedimentation coefficient of the aggregate was approximately 16 S, which would be indicative of a dimer. If the 16 S peak is a dimer, then the average molecular weight of the mixture is slightly contradictory with the previously determined diffusion constant. This has not been resolved as yet. Electrophoresis experiments suggest that this heavy component also has enzyme activity.

When *p*-CMB at concentrations from 10^{-3} to 10^{-4} *M* was added to the enzyme in the synthetic boundary cell, a much smaller, very polydisperse peak with an average sedimentation rate of 6 S resulted. It appears then that the enzyme has broken into fragments, the average of which is about half the original size. It would also appear likely that more vigorous methods should be capable of further breaking down the molecule into even smaller particles.

Microbiology

Enzyme and Tracer Studies on Bacterial Metabolism

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CARBONYL ADDITION TO NICOTINAMIDE ADENINE DINUCLEOTIDE IN FROZEN SOLUTION

M. I. Dolin K. B. Jacobson[1]

The nicotinamide adenine dinucleotide (NAD) modification product isolated from frozen alkaline solutions of commercial NAD has now been identified as the oxidized form of NAD-acetone.[2,3] The acetone necessary for the synthesis is present as a contaminant in all commercial preparations of NAD that have been examined. An average commercial preparation contains approximately 0.3 mole of acetone per mole of NAD.[3] It has been shown that at −20°C, almost all the acetone present is converted to NAD-acetone. The initial adduct, an analog of reduced NAD, is autoxidizable in frozen solution and gives rise to oxidized NAD-acetone. By contrast, in liquid solution[4] high concentrations of nucleophile are required to achieve appreciable adduct synthesis.

The ease with which the nucleophilic addition takes place at −20°C has allowed the synthesis of a variety of carbonyl adducts of NAD. In general, the spectral and fluorimetric properties of these compounds are very similar to those reported for oxidized NAD-acetone.[3] Since the class of compounds being considered may have biological importance as either intermediates or inhibitors of pyridine nucleotide-linked dehydrogenases, the properties of the oxidized acetone adduct have been studied in detail. Spectral, optical rotatory, and fluorimetric properties of the compound, already described in part,[2,3] indicate that oxidized NAD-acetone may be a good model for investigating the relation between conformation and chemical structure in pyridine nucleotides. NMR spectroscopy is currently being used for further study of this phase of the problem.

[1]Enzymology Group.

[2]M. I. Dolin and K. B. Jacobson, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp 142–43.

[3]M. I. Dolin and K. Bruce Jacobson, *Biochem. Biophys. Res. Communs.* **2**, 102 (1963).

[4]R. M. Burton, A. San Pietro, and N. O. Kaplan, *Arch. Biochem. Biophys.* **70**, 87 (1957).

FURTHER CHARACTERIZATION OF A NAPHTHOQUINONE ISOLATED FROM *STREPTOCOCCUS FAECALIS*, 10C1

R. H. Baum M. I. Dolin

Introduction. – The isolation of a quinone from the lactic acid bacterium, *Streptococcus faecalis*, which is able to carry out its metabolic processes essentially by a fermentative means (i.e., without

a tricarboxylic acid cycle) provides a unique situation for the study of the role of quinones in the electron transport system of this type of organism.

Results and Discussion. – Evidence has been presented previously[5,6] for the occurrence of a napththoquinone in *S. faecalis*, strain 10C1. The quinone has now been prepared in a highly purified state by column chromatography and precipitation from absolute ethanol. Both the formula weight and the chromatographic behavior of the quinone indicate that the compound has a side chain of 35 to 45 carbon atoms. Its spectral and chemical properties have been compared with those of synthetically prepared mono- and disubstituted naphthoquinones. Results of these studies indicate that the compound isolated from *S. faecalis* is a monosubstituted 1,4-naphthoquinone containing at least one double bond in the side chain, in a position β to the quinonoid ring. The *S. faecalis* quinone is thus unlike any quinone previously isolated from natural sources. Results of preliminary experiments carried out to determine the effect of oxygen tension on the yield of quinone in growing cultures show that fermentatively grown cells contain about the same amount of quinone as cells grown with vigorous aeration. Based on the borohydride assay of crude lipid extracts, the yield is 2×10^{-4} micromole per milligram of dry weight of cells – about equal to that found in *Escherichia coli*[7] and about one-tenth that reported for *Mycobacterium phlei*.[7]

Experiments are in progress to further characterize the quinone and to determine its metabolic function in the microorganism and its distribution in nature.

MALIC-LACTIC TRANSHYDROGENASE: ENZYME PURIFICATION AND PROPERTIES

E. F. Phares Mary V. Long

A single enzyme from *Micrococcus lactilyticus* (*Veillonella gazogenes*), which is a coupled malic and lactic transhydrogenase, has been reported previously.[8,9] Malate and pyruvate are converted reversibly to oxalacetate and lactate respectively. The enzyme has been further purified, to about 20-fold over the original extract, by chromatography on DEAE-cellulose, Sephadex, and DEAE-Sephadex. Additional purification steps, such as electrophoresis and chromatography on hydroxyl apatite, do not result in higher purification. Protein and activity curves on these preparations are virtually identical. In the analytical centrifuge, a single peak is obtained, which has an $s_{w,20}$ of 6.37×10^{-13} sec or a molecular weight of about 100,000 to 120,000. Various properties of the enzyme have been studied more extensively. Additional tests for hydrogen carriers which will carry out a partial reaction with only one of the substrate pairs have been unsuccessful. Further specificity studies show the reaction to be restricted to L-isomers of lactate and malate.

MALIC-LACTIC TRANSHYDROGENASE: NATURE OF PROSTHETIC GROUP

M. I. Dolin Mary V. Long
E. F. Phares

Malic-lactic transhydrogenase does not utilize artificial hydrogen donors or acceptors, and no requirement for an added cofactor can be demonstrated.[10] With a highly purified enzyme preparation, described above, it has been shown that the prosthetic group of the enzyme is bound pyridine nucleotide. The transhydrogenase contains both reduced and oxidized DPN, in the ratio 2 to 3 moles of DPNH per mole of DPN.

The native enzyme has a broad absorption band between 320 and 400 mμ. The extent to which this band is bleached by the addition of oxidant

[5] R. H. Baum and M. I. Dolin, *Bacteriol. Proc.* **1963**, p 96.

[6] R. H. Baum and M. I. Dolin, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 146.

[7] A. F. Brodie, *Federation Proc.* **20**, 995 (1961).

[8] E. F. Phares and M. V. Long, *Abstracts of Papers, 130th National Meeting of the Americal Chemical Society, 1956*, p 63C.

[9] E. F. Phares *et al.*, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1956*, ORNL-2155, p 57.

[10] E. F. Phares and Mary V. Long, this report.

(pyruvate) depends on the buffer used and the ionic strength; this suggests that the mechanism of the reaction may change as experimental conditions are altered. These experiments have shown that the absorption band is comprised of at least two different absorbing species, possibly the reduced and oxidized forms of bound DPN.

The native enzyme is approximately ten times as fluorescent as free DPNH. Enzyme fluorescence is quenched 98 to 99% on the addition of pyruvate.

The spectral and fluorescence properties of the enzyme should allow a close examination of the mechanism of hydrogen transfer with this dehydrogenase.

Plant Physiology and Photosynthesis

Plant Physiology		Photosynthesis	
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D. E. Foard	Helen J. Luippold[b]	W. F. Bertsch	C. B. Kincaid
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NONESSENTIALITY OF CELL DIVISIONS FOR CHEMICALLY INDUCED CHANGES IN THE DEGREE OF POLARIZATION OF GROWTH

A. H. Haber D. E. Foard N. Ann Payne

Introduction. – Previous studies with wheat leaves have shown that concurrent cell divisions play no role in maintaining a given degree of polarization of growth, p (i.e., the ratio of the specific growth rate in length to the specific growth rate in width).[1] When $p \neq 1$ this means that cell divisions play no role in a given manner in which shape changes as a function of growth.[1] A more general treatment of morphogenesis must also take into account the fact that the degree of polarization itself can be altered. In this study the effects of certain chemicals, known to alter the polarization of growth, have been compared on leaves of gamma plantlets (i.e., seedlings growing without increase in cell number after gamma irradiation of air-dried grains) and on unirradiated controls.

[1]A. H. Haber, *Am. J. Botany* **49**, 583 (1962).

Results. – That the degree of polarization of growth of the first foliage leaf is independent of cell division during germination of wheat is confirmed by the results in Table 42. (Compare water controls of gamma plantlets with water controls of unirradiated plants.) In this system the degree of polarization can nevertheless be changed by chemical treatments. (Among the unirradiated plants, compare the water controls with the gibberellic acid- and colchicine-treated leaves.) Moreover, either the gibberellic acid- or colchicine-induced change in the degree of polarization is about the same in unirradiated leaves as in gamma-plantlet leaves growing without detectable increases in cell number. (Compare the change in the degree of polarization induced by either chemical in gamma plantlets with the change in unirradiated leaves.) The values of the average degree of polarization, p_{av}, calculated in Table 42 must be considered approximations, since the initial dimensions (x_a, y_a) are necessary for calculation of p_{av}, but cannot be measured with accuracy. Note, however, that, relative to water controls, the gibberellic acid significantly increases leaf length while significantly decreasing

Table 42. Chemically Induced Changes in the Degree of Polarization of Growth of First Foliage Leaf of Wheat Seedlings

Material Studied	Chemical Treatment[a]	Number of Leaves Measured	Leaf Length,[b] y_b (mm)	Leaf Width,[b] x_b (mm)	Average Degree of Polarization[c] (p_{av})
6-day-old unirradiated plants	None (water controls)	70	80.00 ± 1.54	3.82 ± 0.06	4.2
	2.4×10^{-4} *M* gibberellic acid	50	125.44 ± 3.10	3.19 ± 0.08	5.5
	0.03% colchicine	20	3.32 ± 0.26	5.58 ± 0.28	1.1
9-day-old gamma plantlets (1000 kr)	None (water controls)	70	12.71 ± 0.61	2.54 ± 0.80	4.1
	2.4×10^{-4} *M* gibberellic acid	50	21.48 ± 0.80	2.28 ± 0.06	5.6
	0.03% colchicine	20	2.78 ± 0.28	4.08 ± 0.26	1.2

[a]Grain was sown and seedlings were kept in solutions indicated.

[b]Data refer to means and 95% confidence limits. Width was measured at the widest part of the leaf.

[c]The p_{av} was calculated as $(\log y_b - \log y_a)/(\log x_b - \log x_a)$. The initial dimensions of the leaf, corresponding to the primordium in the mature grain, were taken as $y_a = 0.66$ mm and $x_a = 1.23$ mm.

leaf width, and that colchicine significantly decreases leaf length while significantly increasing leaf width. Any chemical treatment which, relative to water controls, increases one dimension while decreasing the other must have altered p_{av} and hence the instantaneous values of p also. We can conclude, therefore, that these chemically induced changes in the degree of polarization, both in gamma plantlets and in unirradiated plants, are highly significant in a statistical sense.

Discussion. – Increases and decreases in the degree of polarization of growth are well-known effects of gibberellic acid and colchicine respectively. The results reported here indicate independence of these changes in the degree of polarization from cell division (including independence from the well-known effects of colchicine on the mitotic spindle). From a more general point of view, the results suggest that the concurrent cell divisions which normally accompany growth need play no role in changing the degree of polarization of growth. Such a conclusion is consistent with our earlier conclusion that these cell divisions similarly play no role in maintaining a given degree of polarization of growth.[1]

AN ANALYSIS OF THE APPARENT DIFFERENCE IN SENSITIVITY TO MANNOSE OF EMBRYOS EXCISED FROM IRRADIATED (500 kr) AND UNIRRADIATED WHEAT GRAINS

T. J. Long N. Ann Payne

Introduction. – Previous reports have described effects of the addition of galactose, xylose, ribose, and arabinose to glucose media supporting growth, in darkness, of roots, leaves, and coleoptiles of intact embryos excised from irradiated (500 kr) and unirradiated wheat grains. For these four compounds and three organs, there was only one instance found in which growth of gamma plantlets (i.e., the irradiated plants, which grow without detectable cell division) and unirradiated controls show differential sensitivity to a carbohydrate: addition of galactose greatly reduced growth rate of the first leaf in unirradiated plants but had no significant effect on leaf growth of gamma plantlets.[2] Preliminary results concerning addition of mannose to the glucose medium suggested other differences in response of gamma plantlets vs unirradiated controls.[2] This report

[2]T. J. Long, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p 149.

is concerned with the effects of addition of mannose on the time course of growth of the different organs of gamma plantlets and unirradiated controls.

Results. – Addition of various concentrations (1 to 5 g/liter) of mannose to 15 g/liter of glucose showed no detectable difference in sensitivity of root growth to mannose resulting from the 500-kr irradiation; however, sensitivity of leaf and coleoptile growth to mannose appeared to be decreased by the irradiation. Examination of the time course of growth of unirradiated seedlings showed that both 3 and 5 g/liter of mannose delayed the onset of growth without appearing to reduce growth rate once growth had commenced. Examination of the time course of growth of gamma plantlets showed that mannose at 3 g/liter had no effect on either onset or rate of growth, whereas 5 g/liter almost completely prevented any root growth and delayed the onset of growth of the coleoptile and leaf.

Discussion. – Mannose inhibition, when present in unirradiated controls, may be attributed to a delay in the onset of growth. With respect to this delay, resulting from lower concentrations of mannose, gamma plantlets are less sensitive than are unirradiated controls. At higher concentrations of mannose, various other effects, more difficult to interpret, also occur. Among the carbohydrates we have studied, only galactose produces different effects on growth rates of gamma plantlets vs unirradiated controls in a manner uncomplicated by differential actions on delaying the onset of growth.

PHOTOSYNTHESIS

W. A. Arnold W. F. Bertsch J. R. Azzi

The process by which higher plants convert energy of visible light into chemical oxidation and reduction potential is now known to involve two photoreactions which are linked by enzymes. This initial process of photosynthesis produces adenosine triphosphate and reduced pyridine nucleotide, these two high-energy compounds being utilized to provide energy for reduction of CO_2 to sugars in enzymatic dark reactions of the Calvin cycle. Earlier work from this laboratory has shown that the initial steps of photosynthesis include purely electronic processes as well as enzymatic reactions and that energy conversion processes within the photosynthetic apparatus may be studied by means of the delayed light emission of living plants.

The photosynthetic delayed light is emitted from the singlet-state transition of functionally active chlorophyll. It is a complex phenomenon which is intimately associated with enzymatic reactions and has decay characteristics of untrapping of electrons in a semiconducting crystal. The presence of two photosynthetic pigment systems can be deduced from the different decay curves produced on excitation of the different pigment systems. Delayed light emission at times longer than 10^{-2} sec appears to reflect back reactions of the initial steps of photosynthesis. Emission at faster times seems to measure enzymatic utilization of high-energy electrons from a chlorophyll system during the forward reaction of photosynthesis. We are now stressing experiments at the fast times in an attempt to clarify the physical processes involved in photosynthetic quantum conversion.

In collaboration with J. B. Davidson of the ORNL Instrumentation and Controls Division, we have continued the development of apparatus to measure delayed light at very short times after illumination. We have now reached 5×10^{-5} sec, which is 20 times faster than any previously published value. The decay at this time indicates that earlier electron processes could be observed by measuring the emission at still faster times, and we are considering the feasibility of faster measurements.

A number of photosynthetic poisons have been utilized to provide support for our contention that the fast delayed light emission reflects flow of electrons out of a chlorophyll system during the forward reaction of photosynthetic energy conversion. The effect of poisons on the delayed light emission from 1 to 20 msec was studied in detail. Cyanide (10^{-9} to 10^{-2} *M*) inhibits only the dark reactions of CO_2 reduction, and we found no effect on the fast delayed light emission. 2,4-Dinitrophenol (10^{-8} to 10^{-4} *M*) also had no effect on delayed light emission when used at concentrations which inhibit only respiratory phosphorylation. 2,4-Dinitrophenol at 10^{-3} *M* inhibits all phosphorylation, and at this high concentration both delayed light and fluorescence were extinguished. Azide (10^{-5} to 10^{-1} *M*) is known to act on an enzymatic step which is not close to a

photoreaction but which is involved in quantum conversion, and this compound inhibited delayed light without changing the shape of the decay curve. A number of poisons which act very close to photochemical steps were tested. The effects of these compounds on delayed light emission fall into two categories:

1. Monuron, diuron, *o*-phenanthroline (all at 10^{-7} to 10^{-3} *M*) result in a very flat decay curve, which increases the light emission at 10 to 20 msec by five to ten times. These inhibitors appear to block electron flow out of the chlorophyll system. The flat decay would be due to a large activation energy for the light emission at 20 msec.

2. Hydroxylamine and 2-chloro-6-nitrophenol (both at 10^{-7} to 10^{-3} *M*) result in an exponential decay curve, with increased delayed light at 1 to 3 msec, and decreased delayed light at longer times. Although this result implies a different mode of action of these poisons, we do not yet understand their action in any detail.

Preliminary experiments have been carried out on the 1- to 20-msec delayed light emission of chloroplasts which were performing the Hill reaction (photoproduction of oxygen) under various conditions. The purpose of these experiments was to determine the effect on delayed light of electron acceptors which couple to the Hill reaction at various redox potentials. The implication is that the various Hill oxidants couple to different enzymes in the first-step process. With tripyridine nucleotide ($E_0' = +0.282$ ev) as the Hill oxidant, the light emission was half its value with no Hill oxidant. Since reduction of tripyridine nucleotide implies that the complete photosynthetic energy conversion apparatus was functioning, it might be expected that fewer electrons would be available to produce delayed light. On the other hand, the delayed light at 1 msec was increased when 1,2-naphthoquinone ($E_0' = -0.18$ ev) or 2,6-dichlorophenol indophenol ($E_0' = -0.25$ ev) were used as Hill oxidants. At times longer than 5 msec all Hill oxidants reduced the delayed light. At 1 msec, the oxidant with lowest potential increased the delayed light more than dyes with higher potentials. These results can be explained in terms of a partial functioning of the initial steps of photosynthesis when Hill oxidants with negative potentials are coupled to the reaction.

Biophysics

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OVERLAP OF PHOTOREACTIVATION AND LIQUID HOLDING RECOVERY IN *ESCHERICHIA COLI* B

Amleto Castellani John Jagger
R. B. Setlow

Some bacteria can recover from 2537 A ultraviolet (uv) damage by a postirradiation treatment at longer wavelengths (photoreactivation[1]) or by postirradiation holding of the cells in a liquid (liquid holding recovery[2]). If *Escherichia coli* B in the log phase of growth is inactivated by uv to about 10% survival, we find that, if the cells are then given an optimum photoreactivation treatment (3650 A), no further recovery is obtained upon holding in phosphate buffer at 23°C. Also, if such cells are given an optimum holding treatment, no further recovery is obtained by illuminating with photoreactivating light. We conclude that, at these survival levels in these cells, (1) liquid holding recovery acts only on photoreactivable lesions and (2) liquid holding recovery may be potentially capable of acting on all the photoreactivable lesions.

[1]J. Jagger, pp 352–77 in *Radiation Protection and Recovery* (ed. by A. Hollaender), Pergamon Press, London, 1960.

[2]R. B. Roberts and E. Aldous, *J. Bacteriol.* **57**, 363 (1949).

PMR STUDIES OF POLYPEPTIDE SOLUTIONS

R. H. Dinius

Introduction. – It has previously been shown that a large amount of structure could be induced in the PMR spectra of ribonuclease and lysozyme by recording the spectra at elevated temperatures (>37°C for lysozyme and >58°C for ribonuclease). In contrast to this it was found that chymotrypsin precipitated from solution without the PMR spectra developing structure in the observed broad envelope.[3] These changes have been rationalized in terms of conformational changes in the protein

[3]R. H. Dinius, R. R. Becker, and H. G. Jones, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, pp 190–91.

molecule with subsequent development of configurational freedom and changes in the solute-solvent interactions. To explain these changes more fully and to help explain why the room-temperature PMR spectra is a broad envelope, model polypeptide systems are being studied. Poly-L-lysine, poly-L-glutamic acid, and poly-L-proline are being used because they have physical-chemical properties comparable to the above proteins and there is a large amount of information available about their physical-chemical behavior gathered by other techniques.

The PMR spectra of the lysine and glutamic polypeptides are being recorded as functions of temperature and pH. The pH range for each of these polypeptides extends from the fully ionized to the fully un-ionized form. The temperature range extends from 15 to 75°C. The poly-L-proline is studied in neutral solutions at temperatures less than 30°C.

Methods. – The three polypeptides are dissolved in D_2O, and the lysine and glutamate are transferred to D_2O phosphate buffer systems. Each of the solutions is lyophilized to dryness and redissolved in D_2O twice. This treatment sufficiently reduces the residual solution proton so that the corresponding PMR signal does not obscure other proton bands of interest. The 60-Mc Varian high-resolution spectrometer is used to record the spectra.

Results. – The PMR spectra of poly-L-lysine shows three main bands; poly-L-glutamate and poly-L-proline show two main bands each. The relative separation and bandwidth at half maximum height are measured. Data are relatively complete for poly-L-lysine only, for which the separation of the $(CH_2)_3$ and CH_2 bands is the same in the polymer and the monomer within experimental error (1%). The general shape of the bands is independent of pH but varies differently for the three bands with temperature. The $(CH_2)_3$ band shows no indication of developing the structure found in the monomer, whereas the CH and CH_2 bands do. The PMR absorption bands of poly-L-lysine are broadened further at 75°C than at room temperature.

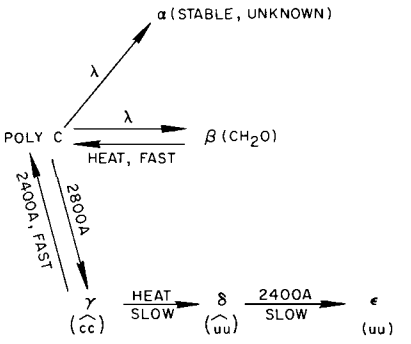
Conclusions. – Conclusions suggested by preliminary analysis of the spectra are (1) there is no appreciable amount of electron delocalization on formation of the polymer from the monomer, (2) the CH_2–CH_2 bond loses almost all rotational freedom whether in the α helix form or random form, and (3) the barrier to this rotation is not overcome prior to the conversion of the polypeptide to the β configuration.

THE PHOTOCHEMISTRY OF POLYCYTIDYLIC ACID

J. D. Griffith R. B. Setlow

Introduction. – The photochemistry of polyC was studied in an attempt to determine the nature and efficiency of production of its photoproducts. A comparison of these results with those obtained for polyT[4] and polyU[5] should indicate the relative importance of these pyrimidines in the biological effects of uv irradiation. Photoproducts have been tentatively identified by their absorption and action spectra and their thermal stability.

Results. – The following reaction scheme fits the data. Identifications are given in parentheses. Reaction $\gamma \rightarrow \delta$ was proposed by D. Weinblum.[6]



The cross sections for production of β and γ are equal and much greater than that for α. Thus little α is seen at low doses but, because the other reactions are reversible, the proportion of α increases with dose. The mean lifetimes at 37°C of CH_2O and $\widehat{CC}$ were 20 and 60 min. Much lower doses are needed to split $\widehat{CC}$ than any of the other known dimers. The 1/e doses at 2400 A for $\widehat{CC} \longrightarrow CC$ and $\widehat{UU} \longrightarrow UU$ were 1.5×10^3 and 1.4×10^4 ergs/mm^2. Initial experiments on the polymerase priming activity of denatured *M.*

[4]R. A. Deering and R. B. Setlow, *Biochim. Biophys. Acta* **68**, 526 (1963).

[5]P. A. Swenson and R. B. Setlow, *Photochem. Photobiol.*, in press.

[6]Cited by A. Wacker in *Progr. Nucleic Acid Res.* 1, 369 (1963).

lysodeikticus DNA (a high C DNA) were in agreement with the predictions of the above reaction scheme.

A POSTULATED MECHANISM FOR PHOTOPROTECTION IN BACTERIA

John Jagger W. C. Wise
R. S. Stafford

Introduction. – Some bacteria show less 2537 A ultraviolet (far uv) killing if given a preirradiation treatment with 3100 to 3800 A (near uv) radiation. This is called photoprotection[7] (PP). The present studies lead to a hypothesis of the mechanism of action of this phenomenon. Preliminary data were previously reported.[8]

Results and Discussion. – It is known that the near uv will induce a division delay of *Escherichia coli* in nutrient broth.[9] We irradiated cells of *E. coli* B at 3341 A and then observed their growth on nutrient agar by phase-contrast microscopy. Doses of near uv that produce good PP were found to induce a growth delay of about 1 hr. After the cells started growing, however, there was an additional division delay, so that filaments were formed. Eventually, about 2 hr after plating, division resumed, resulting in the breaking off of normal cells from the filaments to produce colonies. Thus, the near uv produces both growth delay and division delay on nutrient agar. Further experiments showed that growth delay, as well as the previously reported[9] division delay, also occurs in nutrient liquid.

Growth delay in nutrient liquid was found to follow the same kinetics and to require the same doses as PP. An action spectrum for growth delay in nutrient liquid was obtained. This is shown in Fig. 68, where the points represent the reciprocal of the relative number of incident quanta necessary to produce a doubling of the time required for a fourfold increase in optical density of the culture. The broken line in Fig. 68 represents the action spectrum for PP from killing in the same organism. The action spectrum for PP is corrected at the lower wavelengths for killing effects of the near uv alone, which would partially mask PP; but the spectrum for growth delay is not corrected for killing, since cells killed by moderate doses appear to grow initially as well as surviving cells (that form colonies). These action spectra are identical, within experimental error, showing a single peak at 3380 A.

These similarities in (1) doses required, (2) kinetics, and (3) action spectra lead us to conclude that one of the early steps in PP is the production of a growth-division delay.

How does near uv produce this delay? Previous work[8,10] indicated that the PP photon does not

[7] J. Jagger, *Radiation Res.* **13**, 521 (1960).

[8] J. Jagger, W. C. Wise, and R. S. Stafford, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p 183.

[9] A. Hollaender, *J. Bacteriol.* **46**, 531 (1943).

[10] J. Jagger and R. S. Stafford, *Photochem. Photobiol.* **1**, 245 (1962).

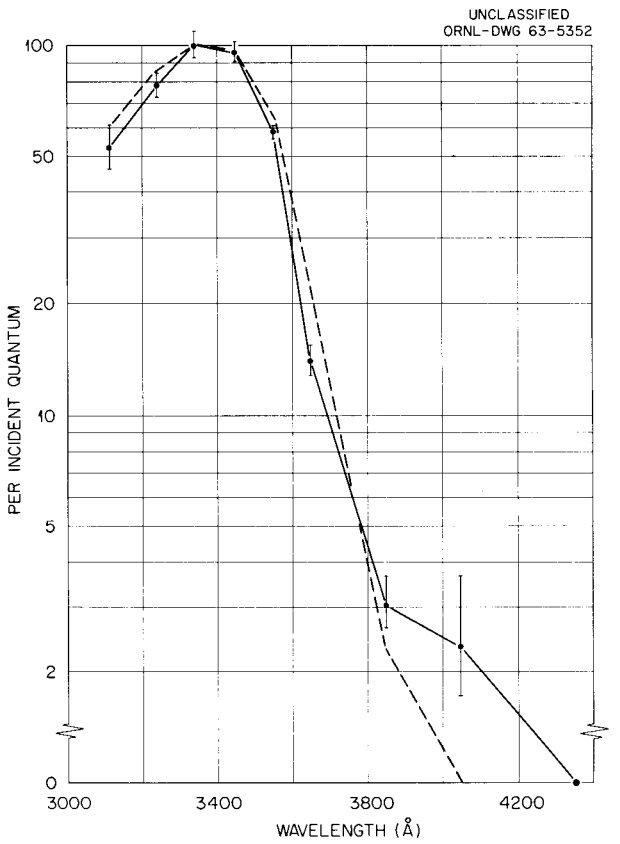


Fig. 68. Action Spectrum for Growth Delay of *E. coli* B in Nutrient Broth. Relative efficiency per incident quantum is plotted on a logarithmic scale vs wavelength. Errors shown are standard deviations of the mean. Each point represents at least three complete growth experiments. Broken line is the action spectrum for photoprotection of *E. coli* B, corrected for killing (from Jagger and Stafford[10]).

act on nucleic acid. We then supposed that it might be inactivating reduced nicotinamide-adenine dinucleotide (NADH), whose absorption spectrum is very similar to the action spectrum for PP. However, we find that this substance is relatively photostable. Recent work by Kashket and Brodie[11] shows that near uv can depress oxidative metabolism in *E. coli* and that it also inactivates respiratory quinones. We find that a vitamin K_2 (a naphthoquinone required by *E. coli* W for oxidative metabolism[11]) is largely destroyed by doses of 3341 A radiation that give good PP. This quinone has an absorption spectrum that fits very well the action spectrum of PP. We suggest, therefore, that photoprotecting doses of near uv radiation inactivate respiratory quinones inside cells. This lowers the metabolic activity of the cells, causing a greatly depressed growth rate and consequent division delay. The cell eventually recovers completely, since the enzymes that produce the quinones are still intact.

Thus, we have a good hypothesis as to why near uv produces growth-division delay. But why should this delay help cells to recover from far uv damage? First, it must be noted that far uv alone produces growth delay. If the delay induced by near uv is to help the cells, it ought to be greater than that produced by far uv alone. Figure 69 shows that this is indeed so. A dose of near uv producing good PP causes a greatly increased growth delay after far uv irradiation.

How might such an induced growth-division delay help the cells to recover from uv damage? We know that cells have recovery mechanisms other than photoreactivation, some of which are called "host-cell reactivation."[12] A division delay could permit more time for such "dark" recovery systems to repair the far uv damage to the cell's nucleic acid before that nucleic acid replicated and thus confirmed the damage.

Is only nucleic acid damage involved in this repair that a photoprotecting treatment provides for? One reason for believing so is that only damage to a self-replicating molecule, such as DNA, would be expected to be irreversible after cell division. Another reason is that other experiments have shown complete overlap in *E. coli* B of PP and liquid holding recovery[2] and of photoreactivation and liquid holding recovery.[13] Therefore, one expects that PP would operate only on photoreactivable damage. Such damage occurs only in nucleic acids.[1]

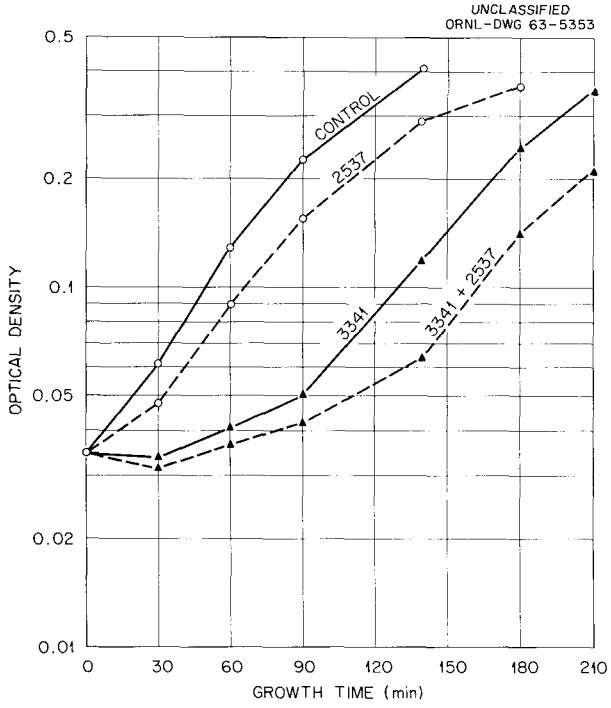


Fig. 69. Growth Delay of *E. coli* B in Nutrient Broth. Optical density on a logarithmic scale is plotted against growth time at 36°C. Circles and solid line show controls. Circles and broken line show effect of a lethal dose of far uv (2537 A, 150 ergs/mm^2, 8% survival). Triangles and solid line show effect of an optimally photoprotecting, but relatively nonlethal, dose of near uv (3341 A, 410,000 ergs/mm^2, 80% survival). Triangles and broken line show effect of the near uv treatment followed by the far uv treatment (60% survival). This is a typical single experiment.

E. coli B_{s-1} (Hill) does not have the capacity for host-cell reactivation.[12] One would therefore expect that this strain could not profit from a division delay induced after far uv irradiation and, therefore, that it would not be photoprotectable. In agreement with this prediction, our tests of this organism show no evidence whatsoever of PP.

[11] E. R. Kashket and A. F. Brodie, *J. Biol. Chem.* **238**, 2564 (1963).

[12] W. Harm, *Z. Vererbungslehre* **94**, 67 (1963).

[13] A. Castellani, J. Jagger, and R. B. Setlow, this report.

DECAY CHARACTERISTICS AND ENERGY OF ACTIVATION OF FREE RADICALS INDUCED IN CRYSTALLINE AMINO ACIDS BY IONIZING RADIATION

M. L. Randolph Mitzi G. Miller

Introduction. – Radiation-induced free radicals in many crystalline amino acids are well known to disappear very slowly at room temperature. The mechanism of decay or recombination of unpaired electron spins might be (1) by abundant and relatively mobile electron donors or receptors, which would imply first-order kinetics, or (2) by direct interactions between seemingly immobile free radicals detectable by electron spin resonance (ESR), which would imply second-order kinetics. If either kinetic order is dominant, decay measurements at constant temperature should reveal the order. Furthermore, if curves are taken at several temperatures for which the same order or mechanism is dominant, the energy of activation associated with the mechanism involved may be determined from an Arrhenius plot.

Materials and Methods. – Samples of four commercially obtained amino acids (glycine, DL-alanine, glutamic acid, and DL-leucine) and one polypeptide (glycyl-glycine) were given x-ray doses of the order of 50 Mrads by a 90-kvp machine with beryllium window. The free-radical concentrations of weighed samples in air were then repetitively measured at room temperature with an ESR spectrometer and an analog computer. Between measurements, spaced a day or more apart, the samples were kept at various constant temperatures[14] from 4 to 120°C. A standard DPPH sample and a mixed ultramarine blue and DPPH sample were used almost daily to check instrument stability. On all but the leucine, two control sets of measurements were made at 90°C: (1) samples evacuated to a few microns pressure overnight and then sealed and (2) nonirradiated samples stored in air.

Results. – From 23 to 120°C, second-order kinetics holds for glycine, alanine, glycyl-glycine, and glutamic acid (except at 120°C where the material turned dark brown and very rapid decay by first-order kinetics occurred). For leucine, second-order kinetics obtains at 4 and 23°C, first-order at 70°C, and either may obtain at 50°C. The evacuated samples at 90°C decay essentially as those stored in air. The unirradiated controls gave, at most, only tiny signals. Using the second-order reaction constants, Arrhenius plots were made and the activation energies were calculated (Table 43).

To make a single display of decay at different temperatures, the integrated form of second-order kinetics was used:

$$\frac{1}{N_t} - \frac{1}{N_0} = K(\theta)\,t \ , \qquad (1)$$

where N's are concentrations at times zero and t, and $K(\theta)$ is the reaction coefficient at absolute temperature θ plucked off the linear fit of the Arrhenius plot. To spread out the Kt scale, one estimates N_0 and plots $(1/N_t - 1/N_0)$ vs $K(\theta)\,t$ on log-log paper. Figure 70 is such a plot.

[14] A set of samples stored at 23°C were given about 16 megarads with a gamma ray source five years ago and had been variously measured meanwhile.

Table 43. Activation Energies in Polycrystalline Materials

Material	Temperature Range (°C)	Energy of Activation (ev/molecule)	Energy of Activation (kcal/mole)
Glycine	4–120	0.53	12.2
Alanine	4–120	0.85	19.5
Glutamic acid	4–90	0.26	6.0
Glycyl-glycine	4–120	0.47	10.8

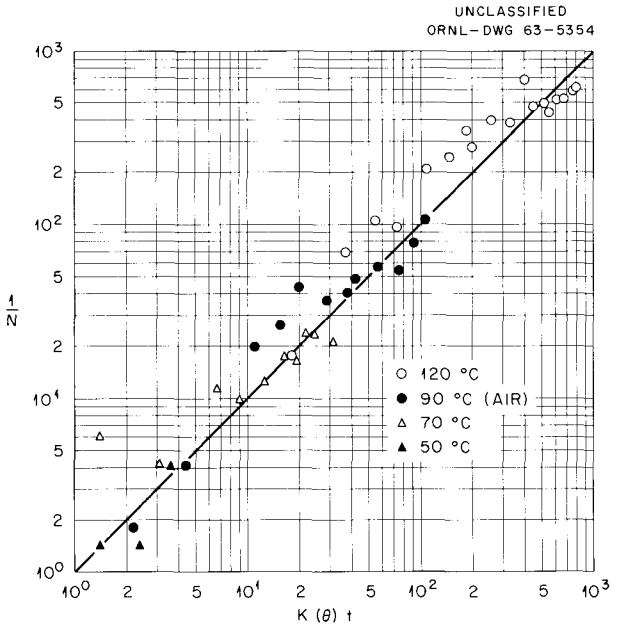


Fig. 70. Plot of Free Radical Decay at Various Temperatures in Irradiated Alanine. Theory predicts a 45° straight line passing through (1, 1).

Discussion and Conclusions. – Reasonable activation energies were found, but measurements are not yet complete. Some materials have second-order decay at low temperatures and first-order at high temperatures. Some materials decay more rapidly immediately following irradiation. That the kinetics in glycine and glutamic acid at room temperature is greater than first order is supported by earlier observations of more rapid decay, on a logarithmic scale, for heavily irradiated than for lightly irradiated materials. Previously, we[15] and others[16] have plotted decay data for dry seed materials as N vs $1/T$, which may be interpreted as merely an approximation to second-order kinetics when N is much smaller than N_0.

THYMINE DIMERS IN UV-IRRADIATED DNA. I. THEIR EFFECTS ON DNA SYNTHESIS *IN VIVO*

R. B. Setlow P. A. Swenson[17]
J. R. Mather

Introduction. – Dimers between adjacent thymine residues in polynucleotide chains are formed by ultraviolet (uv) irradiation. Such dimers inhibit DNA polymerization *in vitro*[18] and are responsible for a large part of the loss in biological activity of uv-irradiated transforming ability.[19] The enzymatic splitting of the dimers is responsible for the increase in transforming activity produced by photoreactivation.[20] We have determined the effects of uv irradiation on the DNA synthesis of various strains of *Escherichia coli* and on *E. coli* infected with irradiated T7 bacteriophage. The uptake of H^3-thymidine was used as the measure of DNA synthesis.

Results. – Typical results are shown in Fig. 71. *E. coli* B_{s-1} is a sensitive and B/r a resistant strain (defined by colony formation). The uv irradiation of sensitive strains results in the permanent cessation of DNA synthesis after a time that decreases with increasing dose. The incorporation data approximately fit a model in which nucleotide polymerization takes place up to a block and then stops. Because the blocks are photoreactivable, we assume that they are the result of thymine dimer formation. A quantitative comparison of the model with the data of Fig. 71 indicates that 2 ergs/mm^2 of 2650 A produces one block per polymerizing unit, and chromatographic data indicate that 2 ergs/mm^2 produce one dimer per 350-μ strand of DNA. Radioautographic data have been interpreted as showing that the polymerizing unit in *E. coli* is 700 μ long.[21]

DNA synthesis of resistant strains is also inhibited by irradiation, but even at large doses the inhibition is not permanent. The resistant cells go around, split, or discard the blocks to DNA synthesis. It is attractive to suppose that the molecular mechanisms responsible for the recovery of DNA synthesis are also responsible for the radioresistance of these strains.

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[15] M. L. Randolph and A. H. Haber, pp 57–65 in *Effects of Ionizing Radiations on Seeds*, International Atomic Energy Agency, Vienna, 1961.

[16] T. Henriksen and A. Pihl, *Nature* **185**, 307 (1960).

[17] Enzymology Group.

[18] F. J. Bollum and R. B. Setlow, *Biochim. Biophys. Acta* **68**, 599 (1963).

[19] R. B. Setlow and J. K. Setlow, *Proc. Natl. Acad. Sci. U. S.* **48**, 1250 (1962).

[20] J. K. Setlow and R. B. Setlow, *Nature* **197**, 560 (1963).

[21] J. Cairns, *J. Mol. Biol.* **6**, 208 (1963).

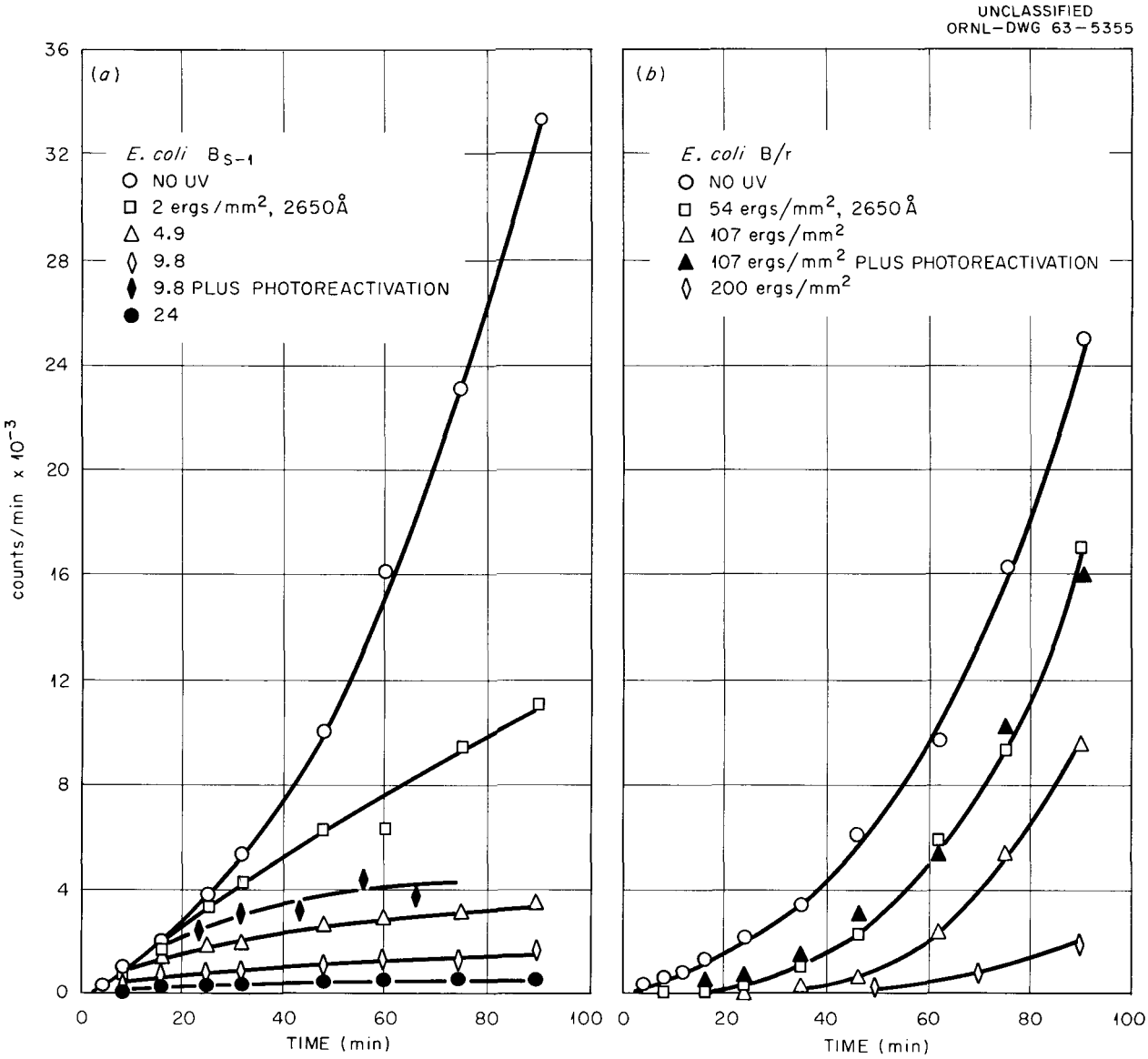

Fig. 71. The Incorporation of H^3-Thymidine into Uv-Irradiated Cultures of *E. coli*. Ten-microliter samples from 37°C incubation mixtures containing at zero time: H^3TdR (2 μg/ml, 6 curies/m*M*), adenosine (200 μg/ml), and bacteria ($\sim 2 \times 10^7$/ml) in M9 medium supplemented with casamino acids. Doubling time of unirradiated cultures, 34 min. The photoreactivating exposure (4000 A, 6×10^5 ergs/mm^2) splits half of the thymine dimers.

If T7 phage DNA is injected into heavily irradiated cells of B_{s-1}, only phage DNA can be polymerized, and H^3-thymidine uptake is now a measure of phage DNA synthesis. Irradiated phage show less DNA synthesis than unirradiated, and 42 ergs/mm^2 represents one block per polymerizing unit. This dose produces one dimer per 17-μ strand of DNA. The length of T7 DNA is thought to be 10 μ (ref 22).

Conclusions. – The following conclusions can be made: (1) The formation of a thymine dimer by uv irradiation produces a block to the *in vivo* polymerization of DNA; (2) the sizes of the polymerizing units of *E. coli* and T7 phage that are inhibited by dimers are within a factor of 2 of

[22] P. F. Davidson and D. Freifelder, *J. Mol. Biol.* **5**, 635 (1962).

those estimated by physico-chemical techniques; and (3) radiation-resistant cells have a molecular mechanism that enables them to repair the blocks. Sensitive strains do not.

THYMINE DIMERS IN UV-IRRADIATED DNA. II. THEIR FATE IN UV-SENSITIVE AND -RESISTANT CELLS

R. B. Setlow W. L. Carrier

Introduction. – Thymine dimers, produced by the uv irradiation of DNA, are important because:

1. They account for a substantial fraction of the lethal effects of uv.
2. They are the only type of radiation "lesion" whose structure is known.
3. It is simple to determine the number of dimers in DNA or cells exposed to uv.

We have used a two-dimensional chromatographic technique to determine the fate of thymine dimers in cells of *E. coli* labeled with H^3-thymidine in an attempt to ascertain the molecular mechanism responsible for the recovery of DNA synthesis in irradiated uv-resistant cells (see previous report).

Results. – The following are our results:

1. Thymine dimers are conserved in both sensitive and resistant cells for times up to 2 hr (in this time resistant cells have resumed DNA synthesis).
2. In *E. coli* B_{s-1} the dimers remain in the trichloroacetic acid (TCA)-insoluble fraction of the cells.
3. In resistant cells the dimers disappear from the TCA-insoluble fraction and appear in the soluble fraction. The times involved are somewhat smaller than those needed for the resumption of DNA synthesis.
4. In *E. coli* B_{s-11} (a sensitive mutant) the dimers and a large fraction of the DNA nucleotides appear in the soluble fraction.

Conclusions. – The repair mechanism responsible for the resumption of DNA synthesis in resistant bacterial strains does not involve the splitting of thymine dimers or the polymerization around them. The repair mechanism acts like a "cut-and-paste" system. The lesions are cut out of the DNA, and the resulting gap is filled by a normal sequence. Obviously, the ability of a cell to form a colony after irradiation will depend on the levels of cutting and patching enzymes in cells. It would be surprising if this mechanism for removing uv lesions were not of importance in recovery of cells from other types of radiation damage and in the general processes of error correction.

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