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SEMIANNUAL PROGRESS REPORT
FOR PERIOD ENDING FEBRUARY 15, 1964

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BIOLOGY DIVISION
SEMIANNUAL PROGRESS REPORT
For Period Ending February 15, 1964

Alexander Hollaender, Director
Stanley F. Carson, Assistant Director

MAY 1964

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Publications and Lectures

PUBLICATIONS

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The proceedings of the Symposium, *Macromolecular Aspects of the Cell Cycle,* held in April 1963 at Gatlinburg were published as a supplement to the October 1963 *Journal of Cellular and Comparative Physiology.*

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Albright, J. F., Takashi Makinodan, and Peter Mazur	Preservation of antibody-producing cells at low temperatures: A method of storage that allows complete recovery of activity	*Proc. Soc. Exptl. Biol. Med.* **114**, 489–93 (1963)
Anderson, N. G. (prepared by)	The Joint National Institutes of Health–Atomic Energy Commission Zonal Centrifuge Development Program: Semiannual report for period January 1–June 30, 1963	ORNL-3502
Anderson, N. G.	Virus isolation in the zonal ultracentrifuge	*Nature* **199**, 1166–68 (1963)
Anderson, N. G., J. G. Green, M. L. Barber, and Sister Francis Clare Ladd	Analytical techniques for cell fractions. III. Nucleotides and related compounds	*Anal. Biochem.* **6**, 153–69 (1963)
Anderson, N. G., C. A. Price, W. D. Fisher, R. E. Canning, and C. L. Burger	Analytical techniques for cell fractions. IV. Reorienting gradient rotors for zonal centrifugation	*Anal. Biochem.* **7**, 1–9 (1964)
Arnold, W. A., and J. B. Davidson	The decay of delayed light at short times	Pp. 698–700 in *Photosynthetic Mechanisms of Green Plants,* Publication 1145, National Academy of Sciences–National Research Council, Washington, D.C., 1963
Barnett, W. E., C. J. Wust, A. G. DeBusk, and D. Frazier	Isolation of high molecular weight DNA from *Neurospora*	*Neurospora Newsletter* **3**, 7 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Baum, R. H., and M. I. Dolin	Isolation of a new naphthoquinone from *Streptococcus faecalis* 10C1	*J. Biol. Chem.* **238**, PC4109–PC4111 (1963)
Becker, R. R.	Discussion after paper by C. B. Anfinsen	Pp. 197–98 in *Brookhaven Symposia in Biology, No. 15: Enzyme Models and Enzyme Structure*, Brookhaven National Laboratory, Upton, New York, 1962
Bell, A. G.	Diploid and endoreduplicated cells: Measurements of DNA	*Science* **143**, 139–40 (1964)
Bender, M. A, and E. H. Y. Chu	The chromosomes of primates	Pp. 261–310 in *Evolutionary and Genetic Biology of Primates*, vol. I (ed. by John Buettner-Janusch), Academic Press, New York, 1963
Bender, M. A, and P. Carolyn Gooch	Chromatid-type aberrations induced by x rays in human leukocyte cultures	*Cytogenetics* **2**, 107–16 (1963)
	Persistent chromosome aberrations in irradiated human subjects	Pp. 424–26 in *The Year Book of Cancer* (1962-1963 Year Book Series) (ed. by R. L. Clark and R. W. Cumley), Year Book Medical Publishers, Inc., Chicago, 1963
Bernlohr, R. W., and G. D. Novelli	Bacitracin biosynthesis and spore formation: The physiological role of an antibiotic	*Arch. Biochem. Biophys.* **103**, 94–104 (1963)
Bertsch, W. F., J. B. Davidson, and J. R. Azzi	Effects of photosynthetic poisons on delayed light in the millisecond time range	Pp. 701–10 in *Photosynthetic Mechanisms of Green Plants*, Publication 1145, National Academy of Sciences–National Research Council, Washington, D.C., 1963
Bollum, F. J.	Intermediate states in enzymatic DNA synthesis	*J. Cellular Comp. Physiol.* **62** (suppl. 1), 61–71 (1963)
Brockman, H. E., and F. J. de Serres	"Sorbose toxicity" in Neurospora	*Am. J. Botany* **50**, 709–14 (1963)
Burger, C. L., W. W. Harris, N. G. Anderson, T. W. Bartlett, and R. M. Kniseley	Virus-like particles in human leukemic plasma	*Proc. Soc. Exptl. Biol. Med.* **115**, 151–56 (1964)
Cameron, I. L.	Organismal regulation of mitotic activity in mice	*Exptl. Cell Res.* **32**, 160–62 (1963)
Cammarano, Piero, Giovanni Giudice, and G. D. Novelli	Binding of amino acyl–sRNA to rat liver polysomes	*Biochem. Biophys. Res. Commun.* **12**, 498–503 (1963)
Christenberry, K. W., J. W. Conklin, A. C. Upton, and G. E. Cosgrove	Induction of cataracts in mice by 4-(*p*-dimethylaminostyryl) quinoline	*Arch. Ophthalmol.* **70**, 250–52 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Cohn, W. E., Viktor Kurkov, and R. W. Chambers	Isolation of pseudouridine from urine	Pp. 135–39 in *Biochemical Preparations*, vol. 10 (ed.-in-chief, G. B. Brown), Wiley, New York, 1963
Congdon, C. C	Biologic mechanisms of protection against radiation	Pp. 578–81 in *Proceedings of the XII International Congress of Dermatology, September 1962, Washington, D.C.*, vol. I (ed. by D. M. Pillsbury and C. S. Livingood), International Congress Series No. 55, Excerpta Medica Foundation, Amsterdam/New York/London/Milan/Tokyo, 1963
	Cell division and cancer (meeting report on the International Symposium on the Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia, July 1–7, (1963)	*Science* **142**, 252–61 (1963)
Cudkowicz, Gustavo, A. C. Upton, G. M. Shearer, and W. L. Hughes	Lymphocyte content and proliferative capacity of serially transplanted mouse bone marrow	*Nature* **201**, 165–67 (1964)
Darden, E. B., and A. C. Upton	Temperature of maximum contraction of mouse collagen fibers as influenced by aging, x-radiation, and breeding status	*J. Gerontol.* **19**, 62–65 (1964)
Davis, M. L., G. E. Cosgrove, W. D. Gude, and Masahide Asano	Liver damage in mice treated with 4-(*p*-dimethylaminostyryl) quinoline, a radiomimetic agent	*Lab. Invest.* **12**, 991–96 (1963)
de Serres, F. J., and B. B. Webber	Recessive lethal mutations resulting from deletion of closely linked loci in balanced heterokaryons of *Neurospora crassa*	*Neurospora Newsletter* **3**, 3–5 (1963)
Doria, Gino	Development of homotransplantation antigens in mouse hemopoietic tissues	*Transplantation* **1**, 311–17 (1963)
Fuccillo, D. A., Jr.	Report of Sixth Annual Institute in Technical and Industrial Communications held July 8–12, 1963, at Colorado State University, Fort Collins	*Bioscience* **14**, 33–34 (1964)
Giudice, Giovanni, and G. D. Novelli	Effect of actinomycin D on the synthesis of DNA polymerase in hepatectomized rats	*Biochem. Biophys. Res. Commun.* **12**, 383–87 (1963)
Goodman, Joan W.	On the origin of peritoneal fluid cells	*Blood* **23**, 18–26 (1964)
	Transplantation of peritoneal fluid cells	*Transplantation* **1**, 334–46 (1963)
Grell, E. H.	Distributive pairing of compound chromosomes in females of Drosophila melanogaster	*Genetics* **48**, 1217–29 (1963)
Haber, A. H., and D. E. Foard	Nonessentiality of concurrent cell divisions for degree of polarization of leaf growth. II. Evidence from untreated plants and from chemically induced changes of the degree of polarization	*Am. J. Botany* **50**, 937–44 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Haber, A. H., T. J. Long, and D. E. Foard	Is final size determined by rate and duration of growth?	*Nature* **201**, 479–80 (1964)
Hollaender, Alexander	Introduction to the proceedings of the conference on Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells	P. v in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, the Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963
	Symposium on Macromolecular Aspects of the Cell Cycle – Introduction	*J. Cellular Comp. Physiol.* **62** (suppl. 1), 198 pp. (1963)
Hollaender, Alexander and S. F. Carson	Biology Division semiannual progress report for period ending August 15, 1963	ORNL-3498
James, T. W., and N. G. Anderson	Continuous recording of cell number in logarithmic and synchronized cultures	*Science* **142**, 1183–85 (1963)
Jenkins, V. K., and A. C. Upton	Cell-free transmission of radiogenic myeloid leukemia in the mouse	*Cancer Res.* **23**, 1748–55 (1963)
Kaji, Hideko, G. D. Novelli, and Akira Kaji	A soluble amino acid–incorporating system from rat liver	*Biochim. Biophys. Acta* **76**, 474–77 (1963)
Karasaki, Shuichi	Studies on amphibian yolk. 5. Electron microscopic observations on the utilization of yolk platelets during embryogenesis	*J. Ultrastructure Res.* **9**, 225–47 (1963)
Kenney, F. T.	Mechanism of hormonal control of rat liver transaminase	Pp. 137–50 in *Advances in Enzyme Regulation*, vol. I (ed. by George Weber), Symposium Publications Division, Pergamon, Oxford, 1963
Kenney, F. T., and F. J. Kull	Hydrocortisone-stimulated synthesis of nuclear RNA in enzyme induction	*Proc. Natl. Acad. Sci. U.S.* **50**, 493–99 (1963)
Kimball, R. F.	The relation of repair to differential radiosensitivity in the production of mutations in *Paramecium*	Pp. 167–78 in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, the Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963
	Repair and differential sensitivity to mutation induction: Summary and synthesis	Pp. 427–41 in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, the Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
	Symposium on repair and differential radiosensitivity at Leiden (meeting report)	*Science* **139**, 56, 58 (1963)
Kretchmar, A. L., and C. C Congdon	The levels of free aspartic acid, glycine, and serine in tissues of bone marrow chimeras	*Transplantation* **1**, 298–305 (1963)
Lindsley, D. L., C. W. Edington, and Elizabeth S. Von Halle	The effect of gametic genotype on the radiation sensitivity of *Drosophila* sperm	Pp. 63–76 in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, the Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963
Lindsley, D. L., and L. Sandler	Construction of the compound-X chromosomes in Drosophila melanogaster by means of the bar stone duplication	Pp. 390–403 in *Methodology in Basic Genetics* (ed. by W. J. Burdette), Holden-Day, Inc., San Francisco, 1963
Löbbecke, E. A., and R. C. von Borstel	Genetically nontransmissible nuclear damage induced by ultraviolet radiation in the wasp Habrobracon	*Genetics* **48**, 1313–22 (1963)
Makinodan, Takashi, and J. F. Albright	Cytokinetics of antibody response	Pp. 99–112 in *Immunopathology, IIIrd International Symposium, La Jolla (California), January 1963* (ed. by Pierre Grabar and P. A. Miescher), Schwabe & Co., Basel, 1963
Mazur, Peter	Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing	*J. Gen. Physiol.* **47**, 347–69 (1963)
Mortimer, R. K., and R. C. von Borstel	Radiation-induced dominant lethality in hapoloid and diploid sperm of the wasp Mormoniella	*Genetics* **48**, 1545–49 (1963)
Parsons, D. F., A. C. Upton, M. A Bender, V. K. Jenkins, E. S. Nelson, and R. R. Johnson	Electron microscopic observations on primary and serially passaged radiation-induced myeloid leukemias of RF mouse	Pp. 464–65 in *The Year Book of Cancer* (1962-1963 Year Book Series) (ed. by R. L. Clark and R. W. Cumley), Year Book Medical Publishers, Inc., Chicago, 1963
Prescott, D. M.	Cell life cycle: Macromolecular aspects (meeting report on the Biology Division Symposium, Gatlinburg, April 8–11, 1963)	*Science* **141**, 547 (1963)
Prescott, D. M., and M. A Bender	Synthesis and behavior of nuclear porteins during the cell life cycle	*J. Cellular Comp. Physiol.* **62** (suppl. 1), 175–94 (1963)
Radomski, M. W., R. A. Wallace, and W. H. Cook	Comparative chromatographic and dissociative behaviour of avian and amphibian lipovitellin	*Biochim. Biophys. Acta* **70**, 600–602 (1963)
Roesel, Hilde A., and A. H. Haber	Studies of effects of light on growth pattern and of gibberellin sensitivity in relation to age, growth rate, and illumination in intact wheat coleoptiles	*Plant Physiol.* **38**, 523–32 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Russell, Liane B.	Radiation hazard	Pp. 156–63 in *Birth Defects* (ed. by Morris Fishbein), J. B. Lippincott Company, Philadelphia and Montreal, 1963
Russell, Liane B., and Clyde L. Saylors	The relative sensitivity of various germ-cell stages of the mouse to radiation-induced non-disjunction, chromosome losses and deficiencies	Pp. 313–42 in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, the Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963
Russel, W. L.	The effect of radiation dose rate and fractionation on mutation in mice	Pp. 205–17 in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, the Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963
Sandler, L., and D. L. Lindsley	The meiotic behavior of tandem acrocentric compound X chromosomes in Drosophila melanogaster	*Genetics* **48**, 1533–43 (1963)
Setlow, Jane K.	The wavelength-dependent fraction of biological damage due to thymine dimers and to other types of leison in ultraviolet-irradiated DNA	*Photochem. Photobiol.* **2**, 393–99 (1963)
Setlow, Jane K., and M. E. Boling	The action spectrum of an *in vitro* DNA photoreactivation system	*Photochem. Photobiol.* **2**, 471–77 (1963)
Setlow, R. B., P. A. Swenson, and W. L. Carrier	Thymine dimers and inhibition of DNA synthesis by ultraviolet irradiation of cells	*Science* **142**, 1464–66 (1963)
Stapleton, G. E., and M. A Bender	The ORNL Space Biology Program, annual report, period ending June 30, 1963	ORNL-TM-720
Stulberg, M. P., and G. D. Novelli	Amino acid–activating enzymes: Methods of assay	Pp. 703–7 in *Methods in Enzymology*, vol. V (ed. by S. P. Colowick and N. O. Kaplan), Academic Press, New York and London, 1962
Suzuki, D. T.	Interchromosomal effects on crossing over in Drosophila melanogaster. II. A reexamination of X chromosome inversion effects	*Genetics* **48**, 1605–17 (1963)
Swartzendruber, D. C., and C. C Congdon	Electron microscope observations on tingible body macrophages in mouse spleen	*J. Cell Biol.* **19**, 641–46 (1963)
Swenson, P. A., and R. B. Setlow	Kinetics of dimer formation and photohydration in ultraviolet-irradiated polyuridylic acid	*Photochem. Photobiol.* **2**, 419–34 (1963)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Upton, A. C.	Biological effects of ionizing radiations	Pp. 199–240 in *International Review of Experimental Pathology*, vol. 2 (ed. by G. W. Richter and M. A. Epstein), Academic Press, New York and London, 1963
	The nucleus of the cancer cell: Effects of ionizing radiation	*Exptl. Cell Res.* Suppl. **9**, 538–58 (1963)
von Borstel, R. C.	Effects of radiation on germ cells of insects: Dominant lethals, gamete inactivation, and gonial-cell killing	Pp. 367–85 in *Proceedings of the Symposium on Radiation and Radioisotopes Applied to Insects of Agricultural Importance*, sponsored by IAEA and held in Athens, Greece, 22–26 April 1963, International Atomic Energy Agency, Kaerntnerring, Vienna, Austria, 1963
	Yeast Genetics Conference (meeting report)	*Science* **142**, 1594 (1963)
von Borstel, R. C., and W. St. Amand	Stage sensitivity to X-radiation during meiosis and mitosis in the egg of the wasp *Habrobracon*	Pp. 87–100 in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, The Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963
Wallace, R. A.	Studies on amphibian yolk. III. A resolution of yolk platelet components	*Biochim. Biophys. Acta* **74**, 495–504 (1963)
	Studies on amphibian yolk. IV. An analysis of the main-body component of yolk platelets	*Biochim. Biophys. Acta* **74**, 505–18 (1963)
Wolff, Sheldon	The kinetics for two-break chromosome exchange and the $^3/_2$ power rule	Pp. 1–10 in *Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells (Proceedings of an International Symposium held at the University of Leiden, the Netherlands, August 15–19, 1962)* (ed. by F. H. Sobels), Symposium Publications Division, Pergamon, Oxford, 1963
Wust, C. J., and G. D. Novelli	Cell-free amino acid incorporation by rat spleen ribosomes	*Arch. Biochem. Biophys.* **104**, 185–91 (1964)
Yamada, Tuneo, and Chinami Takata	An autoradiographic study of protein synthesis in regenerative tissue transformation of iris into lens in the newt	*Develop. Biol.* **8**, 358–69 (1963)
(Members of the Biology Division)	Summary of reports of Bone Marrow Conferences organized by members of the Biology Division, ORNL	Reprint No. 2605

LECTURES

During the period August 1963–February 1964, a total of 211 lectures and speeches were presented by members of the Biology Division. This figure compares with 198 for the corresponding period of 1962–63, and with 182 for the last reporting period. Of the 211 lectures and speeches, 87 were given at meetings of professional societies and 101 on invitation of universities, laboratories, and scientific organizations in the United States or abroad; 23 were presented as part of the Traveling Lecture Program.

For details on speaking activities outside the continental United States, see section "Foreign Travel." Educational activities are described in "Education."

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I.	Genetic analysis of radiation response in bacteria	1. A and M College of Texas, College Station 2. Duke University, Durham, N.C. 3. M. D. Anderson Hospital, Houston, Tex. 4. St. Augustine's College, Raleigh, N.C. 5. University of Texas, Galveston Branch
	Genetic analysis of radiation sensitivity in *Escherichia coli* K-12 [*Genetics* **48**, 881 (1963)]	AIBS, Genetics Society, Amherst, Mass.
	Genetic control of radiation resistance	Am. Soc. Microbiol. (Ky.-Tenn. Branch), Knoxville
	Genetic control of radiation sensitivity and cell division	Protection and Recovery Conference, Oak Ridge
	Genetic control of radiation sensitivity in bacteria	1. Dartmouth Medical School, Hanover, N.H. 2. Rutgers University, New Brunswick, N.J.
Allen, R. C.	Laboratory animal health program, Biology Division, Oak Ridge National Laboratory	14th Annual Meeting, Animal Care Panel, Los Angeles, Calif.
Anderson, N. G.	High resolution automated ion exchange systems	Am. Chem. Soc., 146th Natl. Meeting, Denver, Colo.
	Virus isolation from tissues and from culture fluids	Abbott Sigma Xi Club, Chicago, Ill.
Anderson, N. G. (C. L. Burger and W. W. Harris)	Virus isolation from tissues	Biophysical Society, Chicago, Ill.
Armentrout, H. C.	Chromosomal polymorphism in *Drosophila robusta* populations on Unaka Mountain, Tennessee	Tenn. Acad. Sci., Chattanooga
Arnold, W. A.	The decay of delayed light at short times	Symposium on Photosynthetic Mechanisms of Green Plants, Warrenton, Va.
Barnett, W. E.	Soluble RNA	University of Florida, Gainesville

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Bell, Sandra (and Sheldon Wolff)	Studies of the mechanism of the effect of FUdR on chromosomes [pp. 83–84 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Bender, M. A	Human chromosome abnormalities and their use as a dosimetric tool	Centro di Studi Nucleari della Casaccia, Rome, Italy
	Use of chromosomal aberrations as a dosimetric tool	Atomic Energy Research Establishment, Harwell, England
Bender, M. A (and P. Carolyn Gooch)	Chromosome aberration rates in human somatic cells irradiated *in vivo* [p. 309 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
	Kinetics of aberration loss in the leukocytes of irradiated humans [p. 18 in *Mammalian Cytogenetics Conference*, The National Foundation–March of Dimes, New York, 1963]	Second Mammalian Chromosome Conference, Vergennes, Vt.
Bender, M. A (P. Carolyn Gooch and M. A Bender)	Chromosome aberrations induced in human somatic cells by neutrons	IAEA Symposium on Biological Effects of Neutron Irradiation, Brookhaven National Laboratory, Upton, N.Y.
Bertsch, W. F.	Effects of photosynthetic poisons on delayed light in the millisecond time range	Symposium on Photosynthetic Mechanism of Green Plants, Warrenton, Va.
	Photosynthesis: Biological role, energy relationships, and quantum conversion	UT-AEC Agricultural Research Laboratory Seminar, Oak Ridge, Tenn.
	Recent concepts of quantum conversion in photosynthesis	1. Occidental College, Los Angeles, Calif. 2. Pomona College, Claremont, Calif. 3. University of Georgia, Athens 4. University of Houston, Houston, Tex. 5. University of Texas, Austin
Bollum, F. J.	DNA biosynthesis and its cellular control	Symposium on Molecular Biology, Smith, Kline & French Laboratories, Philadelphia, Pa.
Burger, C. L.	The isolation of virus particles from tissues using zonal centrifugation	Virus Research Group, Charles Pfizer and Son Company, Maywood, N.J.
Burger, C. L. (N. G. Anderson and W. W. Harris)	Virus separation in the zonal ultracentrifuge [*J. Cell Biol.* **19**, 12A (1963)]	Am. Soc. Cell Biol., New York
Cameron, I. L.	The cell cycle of Tetrahymena	University of Massachusetts, Amherst
	Cellular control of DNA synthesis	Brown University, Providence, R.I.
	Macromolecular events leading to cell division in *Tetrahymena pyriformis* after replacement of required pyrimidines [*J. Cell Biol.* **19**, 12A (1963)]	Am. Soc. Cell Biol., New York
Cattanach, B. M.	The inactive-X hypothesis and position effects in the mouse (by title) [*Genetics* **48**, 884–85 (1963)]	AIBS, Genetics Society, Amherst, Mass.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Chu, E. H. Y.	Cytological effects of ultraviolet radiation in mammalian cells	Indiana University, Bloomington
	Cytological effects of ultraviolet radiation on mammalian cells *in vitro*	Stanford University, Stanford, Calif.
	Mammalian chromosome aberrations induced by ultraviolet radiation	Indiana University Medical Center, Indianapolis
	Ultraviolet action spectra for chromosome aberrations of thymidine analog—substituted mammalian cells *in vitro* [p. 85 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Chu, E. H. Y. (T. C. Hsu and D. E. Norby)	Triploid-diploid chimerism in the male tortoiseshell cat [pp. 26–27 in *Mammalian Cytogenetics Conference*, the National Foundation—March of Dimes, New York, 1963]	Second Mammalian Chromosome Conference, Vergennes, Vt.
Cohn, W. E	1. Biosynthesis and metabolism of pseudouridine 2. Some modern approaches to the determination of structure in nucleotides with special reference to pseudouridine	Weizmann Institute, Rehovoth, Israel
	The introduction of elution ion-exchange chromatography into biochemistry and its use in nucleotide separations and nucleic acid investigations (ACS Chromatography Award address)	Am. Chem. Soc., 146th Natl. Meeting, Denver, Colo.
	New nucleotides, structure determination of nucleotides, and chromatography	Takeda Chemical Industries, Osaka, Japan
	Pseudouridine	1. Tokyo University, Tokyo, Japan 2. Faculty of Medicine, Kyoto University, Kyoto, Japan
Congdon, C. C	Secondary disease after bone marrow transplantation	MEND Lecture, University of Oklahoma Medical Center, Oklahoma City
Congdon, C. C (and Alexander Hollaender)	Biological factors in radiation protection and recovery	Inauguration of the Dalat Institute of Applied Nuclear Research, Dalat, Vietnam
Cosgrove, G. E. (and A. C. Upton)	Pathology of the reticulo-endothelial system	Conference on the Pathology of Laboratory Animals, New York Academy of Medicine, New York
Cudkowicz, Gustavo	Attempts to characterize the mouse bone marrow stem cells	The University of Tennessee, Knoxville
Cudkowicz, Gustavo (Michael Bennett and G. M. Shearer)	Evidence for a multipotential stem cell function of the mouse marrow lymphocyte	Protection and Recovery Conference, Oak Ridge, Tenn.
Cudkowicz, Gustavo (and J. H. Stimpfling)	Association of the H-2 locus with the hybrid effect [*Genetics* **48**, 886 (1963)]	AIBS, Genetics Society, Amherst, Mass.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Cudkowicz, Gustavo (A. C. Upton, G. M. Shearer, and W. L. Hughes)	Lymphocyte content and proliferative capacity of serially transplanted mouse bone marrow [*Blood* **22**, 823 (1963)]	6th Annual Meeting, Am. Soc. Hematol., Washington, D.C.
Curtiss, Roy, III	Uv-induced genetic recombination in a partial diploid strain of *E. coli* K-12	Columbia University, New York
Davis, D. G.	Autosomal behavior in claret-nondisjunctional *Drosophila* females [pp. 127–28 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
	Genic control of chromosome behavior in *Drosophila:* claret-nondisjunctional	Protection and Recovery Conference, Oak Ridge, Tenn.
de Serres, F. J.	Analysis of reparable and irreparable mutations in the *ad*-3 region of *Neurospora crassa*	Laboratoire de Genetique Physiologique, Gif-sur-Yvette, France
	1. The effect of different genetic backgrounds on recombination in *Neurospora* 2. The effect of environment on allelic complementation in *Neurospora*	Goucher College, Baltimore, Md.
	Homology tests on x-ray-induced recessive lethal mutations in the *ad-3* region of *Neurospora crassa* [p. 6 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Doherty, D. G.	Chemical protection against ionizing radiation	University of Georgia, Athens
	Chemical protection with mixtures of protective compounds	Protection and Recovery Conference, Oak Ridge, Tenn.
	Enzymes – the biological catalysts	1. The University of Tennessee, Knoxville 2. St. Mary's College, South Bend, Ind.
	The pepsin catalyzed hydrolysis of synthetic substrates	Purdue University, Lafayette, Ind.
Finamore, F. J.	A new pyrophosphate ester from undeveloped eggs	Auburn University, Auburn, Ala.
	An unusual ribonucleic acid fraction from undeveloped eggs	1. Cornell College, Mount Vernon, Iowa 2. Drake University, Des Moines, Iowa 3. Grinnell College, Grinnell, Iowa 4. University of Georgia, Athens 5. University of Virginia, Charlottesville
Finamore, F. J. (and Tuneo Yamada)	The acid-soluble RNA of amphibian eggs and embryos [*Abstr. of papers, 145th Meet., Am. Chem. Soc.* (1963), p. 105C]	Am. Chem. Soc., 145th Natl. Meeting, New York
Fisher, W. D. (G. B. Cline and N. G. Anderson)	Density-gradient centrifugation in angle-head rotors [*The Physiologist* **6**, 179 (1963)]	Am. Physiol. Soc. (15th Autumn Meeting), Coral Gables, Fla.
Foard, D. E.	New approaches to plant morphogenesis	Western Kentucky State College, Bowling Green

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Fritz, P. J.	Studies on lactic dehydrogenase	Auburn University, Auburn, Ala.
Gaulden, Mary Esther	Microbeam irradiation of the nucleous and its effects on nucleolar organizer activity and mitosis	Pennsylvania State University, University Park
Gooch, P. Carolyn (and M. A. Bender)	X-ray-induced chromatid-type aberrations in human blood irradiated *in vitro* [p. 22 in *Mammalian Cytogenetics Conference*, The National Foundation—March of Dimes, New York, 1963]	Second Mammalian Chromosome Conference, Vergennes, Vt.
Grell, E. H.	Nonrandom assortment of compound chromosomes with nonhomologous chromosomes in oocytes of *Drosophila melanogaster* [p. 9 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Grell, Rhoda F.	Factors affecting distributive pairing between nonhomologues in *Drosophila melanogaster* [p. 9 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Gude, W. D.	1. Biological effects of radiation on mammals 2. The use of autoradiography as a tool in research	East Tennessee State University, Johnson City
	1. Biological effects of radiation on mammals 2. The use of autoradiography as a tool in research	Greeneville High School, Greeneville, Tenn.
	Biological effects of radiation on mammals (four lectures)	Science Hill High School, Johnson City, Tenn.
Haber, A. H.	An examination of the concepts of "dormancy" and "germination" from studies with lettuce seeds (read for author)	International Symposium on Physiology, Ecology, and Biochemistry of Germination, Greifswald, East Germany
	The inadequacies of the cell theory in explanations of morphogenesis	Washington University, St. Louis, Mo.
	The inadequacy of the cell theory of development	UT-AEC Agricultural Research Laboratory Seminar, Oak Ridge
Hanna, M. G.	An autoradiographic and histologic study of spleen white pulp germinal centers during early intervals of the primary immune response	The University of Tennessee, Knoxville
Hollaender, Alexander	Development of biology work at Oak Ridge National Laboratory	Comitato Nazionale per l'energia Nucleare, Casaccia, Italy
Jacobson, K. B.	Chromatographic behavior of transfer RNA from various species	Purdue University, Lafayette, Ind.
	Interspecies specificity for acceptor RNA	LeMoyne College, Memphis, Tenn.
	Isozymes of lactic dehydrogenase	Medical School, The University of Tennessee, Memphis
	Reaction of *E. coli* S-RNA with heterologous amino acid activating enzymes	University of North Carolina, Chapel Hill

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Jagger, John	1. Cells, viruses, and DNA 2. Is there life on other worlds?	Everett High School, Maryville, Tenn.
	How does ultraviolet radiation cause the death and mutation of a cell?	1. Louisiana Polytechnic Institute, Ruston 2. Northwestern State College, Natchitoches, La.
	Light and life	1. Centenary College of Louisiana, Shreveport 2. Louisiana Polytechnic Institute, Ruston 3. Northwestern State College, Natchitoches, La.
	Photoreactivation and photoprotection	Conference on Molecular Mechanisms in Photobiology, Wakulla Springs, Fla.
Jagger, John (and R. S. Stafford)	Evidence for two mechanisms of photoreactivation in *E. coli* B	Biophysical Society, Chicago, Ill.
Kenney, F. T.	Hormonal control of protein biosynthesis	1. St. Jude Hospital, Memphis, Tenn. 2. University of Florida, College of Medicine, Gainesville 3. Charles F. Kettering Research Laboratory, Yellow Springs, Ohio
	Mechanisms involved in enzyme changes during development	Am. Chem. Soc., 15th Annual SE Regional Meeting, Charlotte, N.C.
Khym, J. X.	Desalting of nucleic acid and its derivatives by metal hydroxide precipitation	Am. Chem. Soc., 146th Natl. Meeting, Denver, Colo.
Kimball, R. F.	Autoradiographic studies on Paramecium	Karolinska Institute, Stockholm, Sweden
	Quantitative cytochemical studies with the ciliate protozoa	Tennessee Polytechnic Institute, Cookeville
	Studies on radiation mutagenesis in microorganisms	11th International Congress of Genetics, The Hague, the Netherlands
Kondo, Sohei	Increase in resistance to DNA damage with genome complexity for uv, P^{32} Decay, and ionizing radiation	Biophysical Society, Chicago, Ill.
Long, T. J.	Some metabolic aspects of sporocarp development in the mushroom *Collybia velutipes*	1. University of California, Los Angeles 2. University of North Carolina, Chapel Hill 3. The University of Tennessee, Knoxville 4. University of Toledo, Toledo, Ohio
Long, T. J. (and A. H. Haber)	Comparisons between the growth of embryos on carbohydrates after excision from irradiated (500 kr) and unirradiated wheat grains [*Plant Physiol.* **38** (suppl.), xxii (1963)]	AIBS, Am. Soc. Plant Physiol., Amherst, Mass.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Luippold, Helen E. (and Sheldon Wolff)	Studies on the sensitivity of chromosomes to radiation [*Genetics* **48**, 916–17 (1963)]	AIBS, Genetics Society, Amherst, Mass.
Makinodan, Takashi	Antibody formation: Theories in light of recent advances (two lectures)	Emory University, Atlanta, Ga.
	Cytogenetics of antibody formation	Duke University, Durham, N.C.
Mans, R. J.	The role of S-RNA in the maize amino acid incorporating system [*Plant Physiol.* **38** (suppl.) vii (1963)]	1. AIBS, Am. Soc. Plant Physiol., Amherst, Mass. 2. University of Maryland, College Park
Mazur, Peter	Basic problems in cryobiology	Cryogenic Engineering Conf., Boulder, Colo.
Mazur, Peter (and M. T. Harkrider)	Flow of water in multicompartmented cells and tissues during freezing	Biophysical Society, Chicago, Ill.
McArthur, W. H.	Oxygen consumption in lethally irradiated mice given bone marrow intravenously	Protection and Recovery Conference, Oak Ridge, Tenn.
McDonald, T. P. (and T. T. Odell, Jr.)	Two mechanisms of $Na_2S^{35}O_4$ labeling of rat blood platelets [*The Physiologist* **6**, 231 (1963)]	Am. Physiol. Soc. (15th Autumn Meeting), Coral Gables, Fla.
Miller, O. L., Jr.	Autoradiography of soluble H^3-thymidine derivatives in single cells [*J. Cell Biol.* **19**, 50A (1963)]	Am. Soc. Cell Biol., New York
Novelli, G. D.	General mechanisms of protein synthesis	The University of Tennessee, Knoxville
Oakberg, E. F.	The effect of cell stage on radiation-induced cell killing and mutation frequency	1. Institute of Genetics, University of Stockholm, Stockholm, Sweden 2. Finseninstitute, Copenhagen, Denmark
	Population dynamics of irradiated type A spermatogonia of the mouse [p. 82 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Odell, T. T., Jr.	1. Acute injury and recovery in mammals after exposure to alpha, beta, gamma, or neutron radiation 2. Review of basic biology. II. Gross anatomy and physiology	ORNL-ORINS Ten-Week Course in Health Physics, Oak Ridge, Tenn.
Odell, T. T., Jr. (T. P. McDonald and D. G. Gosslee)	Size distribution of blood platelets of varying age	12th Annual Symposium on Blood, Wayne State University, Mayo Clinic, Rochester, Minn.
Padilla, G. M.	Synchronization of cell division	The University of Tennessee, Knoxville
Padilla, G. M. (and P. A. Van Dreal)	Radiation-induced division delay in exponentially and synchronously dividing *Astasia longa* [*J. Cell Biol.* **19**, 54A (1963)]	Am. Soc. Cell Biol., New York
Peck, H. D.	Mechanism of sulfate reduction in the sulfate-reducing bacteria	Dartmouth Medical School, Hanover, N.H.
	Sulfur and energy metabolism in the sulfur bacteria	University of Minnesota, Minneapolis

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Popp, R. A.	Regression of (C57BL × 101)F_2 marrows in irradiated F_1 mice	Protection and Recovery Conference, Oak Ridge, Tenn.
Popp, R. A. (Diana M. Popp and Brenda C. Webb)	Amino acid analyses of tryptic peptides of the α-chain of mouse hemoglobin [*Am. Zool.* **3**, 490 (1963)]	AAAS, Am. Soc. Zool., Cleveland, Ohio
Price, C. A. (N. G. Anderson, W. D. Fisher, R. E. Canning, and C. L. Burger)	Reorienting gradient rotors for zonal centrifugation [*Plant Physiol.* **38** (suppl.), xviii (1963)]	AIBS, Am. Soc. Plant Physiol., Amherst, Mass.
Randolph, M. L.	Free radical concentration measurements by electron spin resonance	Symposium on Recent Developments in Research Methods and Instrumentation, Natl. Inst. Health, Bethesda, Md.
Randolph, M. L. (and Mitzi G. Miller)	Decay of radiation-induced free radicals in amino acid crystals	Biophysical Society, Chicago, Ill.
Ritossa, Ferruccio	Meaning and perspectives of chromosome puffs	Syracuse University, Syracuse, N.Y.
Ritossa, Ferruccio (and J. F. Pulitzer)	Aspects of structure of polytene chromosome puffs of *Drosophila busckii* derived from experiments with antibiotics [*J. Cell Biol.* **19**, 60A (1963)]	Am. Soc. Cell Biol., New York
Russell, Liane B.	Evidence from six X-autosome translocations bearing on the single-active X hypothesis [p. 302 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
	Evidence from X-autosome translocations concerning the hypothesis of the single active X chromosome	Western Reserve University, Cleveland, Ohio
	Genetic activity of the mammalian X-chromosome	University of Wisconsin, Madison
	Genetic activity of the X-chromosome in experimental mammals	N. Y. Acad. Sci.—Columbia University joint meeting on Genetics and the Evolution of Man, New York
	The mammalian X chromosome	Albert Einstein College of Medicine, New York
Russell, Liane B. (Mary Nell Cupp McDaniel and Florence N. Woodiel)	Crossing-over within the a "locus" of the mouse [*Genetics* **48**, 907 (1963)] (by title)	AIBS, Genetics Society, Amherst, Mass.
Russell, W. L.	Evidence from mice concerning the nature of the mutation process [Suppl. 7/III of *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
	Genetic effects of radiation	University of Wisconsin, Madison
	Radiation genetics	Marlboro College, Marlboro, Vt.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Sansing, N. G.	Nucleic acid metabolism in germinating maize	1. University of Georgia, Athens 2. The University of Tennessee, Knoxville
Serrano, L. J.	Proposed federal animal care legislation	Oak Ridge High School Biology Club, Oak Ridge, Tenn.
Setlow, Jane K.	Physical photochemistry of nucleic acids	Conference on Molecular Mechanisms in Photobiology, Wakulla Springs, Fla.
Setlow, R. B.	Error mechanisms at the molecular level	Joint Physics Colloquium, University of North Carolina and Duke University, Durham, N.C.
	Role of thymine dimers in the inactivation of cells by ultraviolet radiation	Biophysical Society, Chicago, Ill.
	Thymine dimers, DNA synthesis, and error checking	1. University of Chicago, Chicago, Ill. 2. University of Michigan Medical School, Ann Arbor
Seto, Frank	A quantitative study of homologous splenomegaly in the chick embryo [*Genetics* **48**, 909 (1963)]	AIBS, Genetics Society, Amherst, Mass.
Smith, L. H.	Radioprotective and recovery measures	Armed Forces Institute of Pathology, Washington, D.C.
Stevens, A. Rebecca	Electron microscope autoradiography of DNA and RNA syntheses in *Euplotes eurystomus* [*J. Cell Biol.* **19**, 67A (1963)]	Am. Soc. Cell Biol., New York
Stone, G. E.	DNA synthesis in *Tetrahymena* deprived of essential amino acids [*J. Cell Biol.* **19**, 68A (1963)]	Am. Soc. Cell Biol., New York
Swartzendruber, D. C.	The fate of plasma cells during an immune response	Protection and Recovery Conference, Oak Ridge, Tenn.
Swenson, P. A. (and R. B. Setlow)	Inhibition of macromolecular synthesis in *E. coli* by ultraviolet irradiation	Biophysical Society, Chicago, Ill.
Swenson, P. A. (R. B. Setlow, P. A. Swenson, and W. L. Carrier)	Thymine dimers and the inhibition of DNA synthesis by uv irradiation of cells	Society of General Physiologists, Woods Hole, Mass.
Upton, A. C.	1. Future directions of research 2. Irradiation and rate of degradative changes 3. Life shortening 4. Similarity between late radiation effects' manifestation and the signs and symptoms of aging	Late Irradiation Effects Conference, AEC, Division of Biology and Medicine, Germantown, Md.
	Ionizing radiation in the causation of cancer	Central Ohio Society of Pathologists, Columbus
	1. Pathologic effects of ionizing radiation 2. Radiation-induced life shortening and its relation to aging	Ohio State University, Columbus

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
	Radiation and carcinogenesis	Armed Forces Institute of Pathology, Washington, D.C.
	Radiation effects	University of Pittsburgh School of Medicine, Pittsburgh, Pa
	Radiation hazards to man	Navy Nuclear Science Seminar, Oak Ridge, Tenn.
	Some applications of radiation and radioisotopes to experimental pathology	Am. Assoc. University Pathologists, Chicago, Ill.
Upton, A. C., (M. L. Randolph, K. W. Christenberry, E. B. Darden, and J. W. Conklin)	Relative biological effectiveness of fast neutrons for late somatic effects in mice	IAEA Symposium on Biological Effects of Neutron Irradiation, Brookhaven National Laboratory, Upton, N.Y.
Van Dreal, P. A. (and G. M. Padilla)	Carbon dioxide as a requirement for cytokinesis in temperature-synchronized *Astasia longa*	Biophysical Society, Chicago, Ill.
Volkin, Elliot	Biochemical transformation of bacteriophage DNA	Case Institute and Western Reserve University, Cleveland, Ohio
von Borstel, R. C.	1. Cell killing by radiation 2. Spontaneous and induced mutations in yeast	Greek Nuclear Center, Athens, Greece
	1. Dominant lethality and cell killing by radiation 2. Kinetics of radiation effects on cells 3. Stage sensitivity and species response to radiation in different insects	University of Florida, Gainesville
	Inherited partial sterility in *Habrobracon* [pp. 124–25 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
	Radiation-induced breakage of lampbrush chromosomes *in situ* and *in vitro*	University of California, Berkeley
von Borstel, R. C. (D. M. Prescott and F. J. Bollum)	Cytochemical localization of primer DNA using DNA polymerase [*J. Cell Biol.* **19**, 72A (1963)]	Am. Soc. Cell Biol., New York
von Borstel, R. C. (and R. E. Scossiroli)	Selection experiments after inbreeding in *Habrobracon* [p. 152 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Vu Thi Suu	Lysozyme activity in radiation chimeras	Protection and Recovery Conference, Oak Ridge, Tenn.
Walburg, H. E., Jr. (and Gustavo Cudkowicz)	Studies on cellular turnover in gnotobiotic mice by use of I^{131}-iododeoxyuridine	AIBS Symposium on Recent Advances in Germfree Animal Experimentation, 1959-1962, Amherst, Mass.
Wald, Niel (A. C. Upton, V. K. Jenkins, and W. H. Borges)	The consistent occurrence of an extra and a marker chromosome in mouse granulocytic leukemia [pp. 28–29 in *Mammalian Cytogenetics Conference*, The National Foundation–March of Dimes, New York, 1963]	Second Mammalian Chromosome Conference, Vergennes, Vt.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Wald, Niel (A. C. Upton, V. K. Jenkins, and Sylvia F. Pan)	The consistent occurrence of an extra and a marker chromosome in two passaged mouse post-irradiation granulocytic leukemias [*Blood* **22**, 817 (1963)]	Am. Soc. Hematol., Washington, D.C.
Wallace, R. A.	The formation and structure of yolk granules	The Ottawa Biological and Biochemical Society, Ottawa, Canada
Welshons, W. J. (E. S. Von Halle and B. J. Scandlyn)	Notch pseudoalleles in *Drosophila melanogaster* [pp. 1–2 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Wolff, Sheldon	Biophysical and biochemical processes involved in chromosome exchange	Purdue University, Lafayette, Ind.
	The kinetics for the production of 2-hit chromosomal aberrations and the $^3/_2$ power rule	University of Virginia, Charlottesville
	1. On the doubleness of the chromosome as revealed by combined x-ray and labeling experiments 2. Preliminary experiments on the chemistry of crossing over	Comitato Nazionale per l'Energia Nucleare, Casaccia, Italy
Wolff, Sheldon (and F. J. deSerres)	Preliminary experiments on the chemistry of crossing over [p. 12 in *Genetics Today* (ed. by S. J. Geerts), Pergamon, New York, 1963]	11th International Congress of Genetics, The Hague, the Netherlands
Woodiel, Florence N. (and Liane B. Russell)	A mosaic formed from fertilization of two meiotic products of oogenesis [*Genetics* **48**, 917 (1963)] (by title)	AIBS, Genetics Society, Amherst, Mass.
Yamada, Tuneo	The pattern of macromolecular synthesis during regenerative tissue transformation	Colloquium of the Biological Laboratories, Harvard University, Cambridge, Mass.
Yamada, Tuneo (and Chinami Takata and J. F. Albright)	Lens antigens in transformation of adult newt iris into the lens [*J. Cell Biol.* **19**, 69A–70A (1963)]	Am. Soc. Cell. Biol., New York
Zill, L. P. (and J. X. Khym)	Ion exchange chromatography of sugars with the aid of borate complex formation	Am. Chem. Soc., 146th Natl. Meeting, Denver, Colo.

VISITING LECTURERS

During the six months preceding this report, 37 lectures were given on the Biology Division Seminar Program by guest speakers from scientific institutions and universities in this country and abroad. Lectures were given by scientists from Belgium, Czechoslovakia, England, Germany, Scotland, Sweden, and Switzerland.

SPEAKER	AFFILIATION	SUBJECT
Herman Ainis	Childrens Asthma Research Institute & Hospital, Denver, Colo.	Factors affecting antibody synthesis *in vitro*
W. R. Bryan	Laboratory of Viral Oncology, National Cancer Institute, Bethesda, Md.	Viruses and cancer
John Cairns	Cold Spring Harbor Laboratory of Quantitative Biology, Cold Spring Harbor, N.Y.	Progress in the autoradiography of DNA
Lucien Caro	Institut de Biologie Moleculaire, Laboratorie de Biophysique, Université de Genève, Genève, Switzerland	DNA of bacteriophage lambda
J. W. Daniel	McArdle Memorial Laboratory, University of Wisconsin, Madison	Film – Physarum Reactions controlling the induction of morphogenesis of a slime mold
A. J. S. Davies	Chester Beatty Research Institute, London, England	A variety of chimaeras
A. B. Edmundson	Laboratory of Molecular Biology, University Postgraduate Medical School, Hills Road, Cambridge, England	The chemical aspects of the molecular structure of myoglobin
J.-E. Edström	Department of Histology & Neurobiology, University of Göteborg, Göteborg, Sweden	Microelectrophoretic nucleic acid analysis in the study of nuclear cytoplasmic relations
E. J. Eichwald	Laboratory for Experimental Medicine, Montana Deaconess Hospital, Great Falls	The case of the variegated male
Duane Engelhardt	Systems Engineer, IBM, Knoxville, Tenn.	Selective dissemination of information
D. S. Falconer	Institute of Animal Genetics, Edinburgh, Scotland	Genetics studies of mouse lung-tumors
Reed Flickinger	Department of Zoology, University of California, Davis	Sequential synthesis of RNA in embryonic development
D. MacDonald Green	Department of Chemistry, Harvard University, Cambridge, Mass.	Infection of the *Bacillus subtilis* with DNA isolated from bacteriophage
Niels K. Jerne	Department of Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.	The appearance of antibody forming cells in the spleen of mice after primary experimental antigenic stimulus
G. T. Judson	International Business Machines, Endicott, N.Y.	Separation of formed elements of blood
H. Jung	Kernforschungszentrum Karlsruhe, Institut für Strahlenbiologie, Karlsruhe, Germany	Slow proton irradiation of polyethylene terephthalate
J. L. Key	Department of Botany & Plant Pathology, Purdue University, Lafayette, Ind.	The effect of auxin on nucleic acid metabolism in soybeans
J. R. Maisin	Radiobiology Department, Laboratoires du C.E.N. a Mol-Donk, Mol-Donk, Belgium	Chemical protection of mammalian tissue with special emphases on early changes
John Papaconstantinou	Department of Zoology & Entomology, The University of Connecticut, Storrs	Proteins, RNP and RNA in lens development
James Peacock	Department of Biology, University of Oregon, Eugene	An analysis of chromosome structure

SPEAKER	AFFILIATION	SUBJECT
Robert Perry	The Institute for Cancer Research, Fox Chase, Philadelphia, Pa.	Synthesis of the various types of RNA with reference to cellular location
R. A. Rinaldi	Department of Zoology, University of Tennessee, Knoxville	Microcinematography of amoebae – Film
Pablo Rubinstein	The Mary Imogene Bassett Hospital, Cooperstown, N.Y.	Some immunogenetic problems in the H-2 system of the mouse
Ruth Sager	Department of Botany, Columbia University, New York	What are non-chromosomal genes?
H. L. Segal	Department of Pharmacology, St. Louis University School of Medicine St. Louis, Mo.	Glucocorticoid control of glutamic-alanine transaminase synthesis
D. G. Sharp	Department of Biophysics, University of North Carolina, Chapel Hill	Virus counting methods
Michel Sicard	Department of Microbiology, Western Reserve University, Cleveland, Ohio	Recombination studies with Pneumococcus DNA
Jaroslav Sterzl	Biological Institute, Czechoslovak Academy of Sciences, Prague, Czechoslovakia	Development of specific and non-specific immune reactions
J. H. Stimpfling	The Jackson Laboratory, Bar Harbor, Me.	Some properties of erythrocyte antigens of the laboratory mouse
Heinz Tiedemann	Heiligenberg Institute, Heiligenberg, West Germany	Biochemical aspects of primary induction and determination
Rhodes Troutman	U.S. Department of Agriculture, Plum Island Animal Disease Laboratory, Greenport, Long Island, N.Y.	Biophysical studies on foot and mouth disease virus
W. S. Vincent	Department of Anatomy, University of Pittsburgh Medical School, Pittsburgh, Pa.	Some relations between nucleoli and ribosomes
Evelyn M. Witkin	State University of New York Downstate Medical Center, Brooklyn	Photoprotection, photoreactivation and dark repair in the study of UV-induced mutation in bacteria
R. F. Wood	Solid State Division, ORNL	Application of elementary quantum mechanics to biology
E. A. Wright	Department of Radiology, Stanford University School of Medicine, Palo Alto, Calif.	Dynamics of hair growth in relation to the effects of radiation
B. K. Zimmerman	Committee on Biophysics, University of Chicago, Chicago, Ill.	Cross-linked DNA

SPEAKERS AT PROFESSIONAL MEETINGS, SPRING–SUMMER 1964

Eighth Annual Meeting of the Biophysical Society – N. G. Anderson (coauthors, C. L. Burger and W. W. Harris), John Jagger (coauthor, R. S. Stafford), Sohei Kondo, Peter Mazur (coauthor, M. T. Harkrider), M. L. Randolph (coauthor, M. G. Miller), P. A. Swenson (coauthor, R. B. Setlow), and P. A. Van Dreal (coauthor, G. M. Padilla)

18th Annual Symposium on Fundamental Cancer Research – Sheldon Wolff

Information Meeting on Lens Differentiation – Shuichi Karasaki, Chinami Takata (coauthor, J. F. Albright), and Tuneo Yamada (coauthors, Chinami Takata, S. Eisenberg, and J. Kohonen)

77th Annual Meeting of the American Association of Anatomists – I. L. Cameron and Donald Swartzendruber

17th Annual Biology Research Conference – F. J. de Serres and R. B. Setlow

Association for Research in Ophthalmology Eastern Sectional Meeting – Tuneo Yamada (coauthors, Chinami Takata and J. F. Albright)

Federation of American Societies for Experimental Biology – N. G. Anderson (coauthors, C. L. Burger and H. P. Barringer), W. E. Barnett (coauthor, K. B. Jacobson), F. J. Bollum (coauthors, E. Groeniger and M. Yoneda), Piero Cammarano (coauthors, Giovanni Giudice, Marialuisa Melli, and G. D. Novelli), C. C Congdon (coauthors, M. A. Kastenbaum and D. A. Gardiner), Gustavo Cudkowicz, M. I. Dolin (coauthors, E. F. Phares and M. V. Long), F. J. Finamore, Robert Fujimura (coauthor, Paul Kaesberg), Joan Wright Goodman, R. J. Mans, C. G. Mead (coauthor, Elliot Volkin), E. H. Perkins, F. Pochon (coauthors, A. M. Michelson, M. Grunberg-Manago, L. Dondon, and W. E Cohn), Toshihiko Sado (coauthors, I. Hoppe and P. Nettesheim), W. D. Wicks (coauthor, F. T. Kenney), and G. R. Williams

Association of Southeastern Biologists – Sandra Bell (coauthor, Sheldon Wolff) and J. O. Krivanek

American Society for Microbiology – H. I. Adler (coauthor, Alice A. Hardigree), Roy Curtiss III, D. E. Duggan (coauthor, H. I. Adler), A. P. Harrison, Jr. (coauthor, L. E. Franklin), G. E. Stapleton, E. J. Johnson (coauthor, H. D. Peck, Jr.), R. L. Tyndall (coauthors, K. B. Jacobson and Ernestine Teeter), Elliot Volkin (coauthor, M. H. Jones), Joy P. Williams (coauthors, J. T. Davidson and H. D. Peck, Jr.), and C. J. Wust

Radiation Research Society – H. I. Adler (coauthor, Alice A. Hardigree), G. E. Cosgrove (coauthor, L. H. Smith), F. J. de Serres, J. A. Heddle, A. C. Upton (coauthors, J. W. Conklin and M. L. Randolph), and H. E. Walburg, Jr. (coauthors, E. I. Mynatt and D. M. Robie)

4th International Photobiological Congress – W. F. Bertsch, Mary Esther Gaulden (coauthors, J. G. Carlson and John Jagger), John Jagger (coauthor, R. S. Stafford), Jane K. Setlow (coauthor, M. E. Boling), R. B. Setlow (coauthor, W. L. Carrier), and P. A. Swenson (coauthor, R. B. Setlow)

6th International Congress of Biochemistry – M. I. Dolin (coauthor, K. B. Jacobson), F. T. Kenney (coauthors, W. D. Wicks and D. L. Greenman), Amir Muhammed, F. Pochon (coauthors, A. M. Michelson, M. Grunberg-Manago, L. Dondon, and W. E Cohn), and Elliot Volkin (coauthor, M. H. Jones)

Xth International Botanical Congress – W. F. Bertsch and A. H. Haber (coauthor, D. E. Foard)

FOREIGN TRAVEL

The XI International Congress of Genetics held at The Hague, the Netherlands, September 2–10, was attended by Alexander Hollaender and 15 other Division members. While in The Hague, Dr. Hollaender presided at a meeting of the Council of the International Association for Radiation Research on September 9–11. He attended a meeting of the World Health Organization in Geneva on September 12–14, spoke at the Comitato Nazionale per l'Energia Nucleare in Casaccia, Italy, on "Development of biology work at Oak Ridge National Laboratory," and visited laboratories in Casaccia, Rome, and Naples as a member of the Advisory Committee to the Division of Biology of the Comitato Nazionale per l'Energia Nucleare.

Papers were presented at the Genetics Congress by Sandra Bell, Gale Davis, E. H. and Rhoda Grell, and W. J. Welshons, who was on leave of absence at the Universidad de Chile, Santiago. Papers were also given by the following Division members, who made the additional visits and/or presentations indicated:

M. A Bender attended the Second Conference on the Normal Human Karyotype in London, visited laboratories in Harwell (England), Paris, Padua, Rome, and Naples; gave informal lectures on the same topic as the paper presented at The Hague; and held seminars on "Use of chromosomal aberrations as a dosimetric tool," at the Atomic Energy Research Establishment, Harwell, and "Human chromosome abnormalities and their use as a dosimetric tool," at Centra di Studi Nucleare della Casaccia, Rome.

B. M. Cattanach visited the Institute of Animal Genetics in Edinburgh.

E. H. Y. Chu attended the Second Conference on the Normal Human Karyotype and visited laboratories in France and Sweden for consultation on problems in mammalian genetics, cytology, and radiation and tumor biology.

F. J. de Serres visited laboratories in Edinburgh, Cambridge, Rome, Naples, Copenhagen, and Paris and spoke at the Laboratoire de Genetique Physiologique, Gif-sur-Yvette, on "Genetic analysis of reparable and irreparable mutations in the *ad-3* region of *Neurospora crassa.*"

R. F. Kimball gave a seminar on "Autoradiographic studies on *Paramecium*" at the Karolinska Institute in Stockholm and visited universities in Stockholm, Gothenburg (Sweden), and Edinburgh.

E. F. Oakberg lectured on "The effect of cell stage on radiation-induced cell killing and mutation frequency" at the Institute of Genetics, University of Stockholm, and at the Finseninstitute, Copenhagen, and visited laboratories in Jouy-en-Josas (France) and Rome.

Liane B. and W. L. Russell visited the Galton Laboratory at the University of London.

R. C. von Borstel attended a meeting of yeast geneticists at Gif-sur-Yvette (France); consulted with entomologists at the Greek Nuclear Center, Athens; and presented seminars there on "Cell killing by radiation" and "Spontaneous mutations in yeast." He also consulted with the International Atomic Energy Agency in Vienna on problems of bibliographic indexing of radiation genetics.

Sheldon Wolff presented seminars at the Comitato Nazionale per l'Energia Nucleare, Casaccia, Italy, entitled "On the doubleness of the chromosome as revealed by combined x-ray and labeling experiments" and "Preliminary experiments on the chemistry of crossing over." He also visited laboratories at Harwell, Paris, Saclay, and Copenhagen.

Other Division members engaged in foreign travel were W. E Cohn and C. C Congdon.

W. E Cohn lectured on "Some modern approaches to the determination of structure in nucleotides with special reference to pseudouridine" and "Biosynthesis and metabolism of pseudouridine" at Weizmann Institute, Rehovoth, Israel; on "New nucleotides, structure determination of nucleotides, and chromatography" at the Research Laboratories of Takeda Chemical Industries, Osaka, Japan, and on "Pseudouridine" at the Universities of Kyoto and Tokyo, Japan.

C. C Congdon attended the official inauguration of the Dalat Institute of Applied Nuclear Research in Dalat, Vietnam, and prepared the paper "Biological factors in radiation protection and recovery" for inclusion in the Institute's commemorative publication. He also conferred with foreign investigators at the Institute and at the Saigon Atomic Energy office. He visited the National Institute of Radiobiological Sciences in Tokyo for discussion of experimental pathology and bone marrow transplantation.

R. A. Wallace, who left in August to spend ten months in Ottawa, Canada, in collaborative work between the Division and the National Research Council's Division of Applied Biology, presented "The formation and structure of yolk granules" to the Ottawa Biological and Biochemical Society.

SEVENTEENTH ANNUAL BIOLOGY RESEARCH CONFERENCE

The 1964 Annual Research Conference, The Molecular Action of Mutagenic and Carcinogenic Agents, sponsored by the Biology Division of Oak Ridge National Laboratory, was held at the Mountain View Hotel, Gatlinburg, Tennessee, April 6–9 inclusive. A series of four seminars was given at the Division

during February as a preview. These were presented by R. B. Setlow, A. C. Upton, R. F. Kimball, and W. E. Barnett. The organizing committee, composed of Elliot Volkin, chairman, R. B. Setlow, R. F. Kimball, A. C. Upton, G. D. Novelli, and C. C Congdon, announced the following program:

Monday, April 6

Opening Remarks – Alexander Hollaender

Welcome Address – Alvin M. Weinberg, Director, Oak Ridge National Laboratory

Introduction – Elliot Volkin, Chairman, Organizing Committee

Morning

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

Acridine mutagens and DNA structure – L. S. Lerman, Department of Biophysics, School of Medicine, University of Colorado, Denver

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

Afternoon

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

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

Characterization of mutants in bacteriophage – Sydney Brenner, Laboratory of Molecular Biology, University Postgraduate Medical School, Cambridge, England

Tuesday, April 7

Morning

Chairman – Adolf Wacker, Institut für therapeutische Biochemie, Universität Frankfurt, Frankfurt am Main, Germany

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

The role of purine and pyrimidine bases and their analogues in radiation sensitivity – H. S. Kaplan, Department of Radiology, Stanford University School of Medicine, Palo Alto, California

Afternoon

(Free)

Wednesday, April 8

Morning

Chairman – C. E. Carter, Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

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

The reaction of some mutagenic and carcinogenic compounds with nucleic acids – Peter Brookes and P. D. Lawley, Chester Beatty Research Institute, London, England

Afternoon

Chairman – Louis Siminovitch, Ontario Cancer Institute, Toronto, Canada

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

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, The Salk Institute for Biological Studies, San Diego, California

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

Virus defectiveness and cell transformation in the Rous sarcoma – Harry Rubin, Virus Laboratory, University of California, Berkeley

Summary – Renato Dulbecco

ANNUAL INFORMATION MEETING

The ORNL Advisory Committee for Biology made its annual visit to the Division November 11–13. The committee members are Erwin Chargaff, Columbia University; Rollin D. Hotchkiss, The Rockefeller Institute; Earl R. Stadtman, National Heart Institute; Curt Stern, University of California; and Maxwell M. Wintrobe, University of Utah. Dr. Stern was abroad and could not attend the meeting, and he was represented by Herschel Roman, University of Washington, Seattle. Group leaders and their associates presented a review of current research studies at a conference held the first day of the meeting. The committee members visited individual laboratories and participated in informal discussions on the other two days.

BONE MARROW CONFERENCES

A meeting on Radiation Protection and Recovery was held November 22, at the Holiday Inn, Oak Ridge. This was the latest in the series of bone marrow transplantation and chemical protection conferences begun in 1957 in Oak Ridge; proceedings will be published in *Blood, The Journal of Hematology,* and in the *Biology Division Bone Marrow Booklet, No. 6.*

Meetings planned for 1964 are:

April 13, Chicago, Ill. – Chairman, C. C Congdon

September 7–9 (tentative date), Paris, France – Chairman, Georges Mathé

November 20–21, Houston, Texas – Cochairmen, Daniel Billen and John Trentin

CONFERENCE ON LENS DIFFERENTIATION

A conference on lens differentiation, sponsored by the Biology Division and organized by Tuneo Yamada, is planned for March 20–21. Scientists from several states will present papers covering experimental analysis of lens development and regeneration, ultrastructure of normal and regenerating lenses, and cell biological and biochemical aspects of lens differentiation. Included in the program are papers by three Biology Division investigators.

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 Alexander Hollaender. The first was in Boston, October 14–15, 1963, and the second at the New York Operations Office, February 17–18, 1964.

EDUCATIONAL ACTIVITIES

Student Trainee Program. – Nineteen student trainees have been selected to spend the summer of 1964 in the Division. These are predominantly from small undergraduate colleges in the South.

Small College Program. – Three members of the Biology Division spoke at small colleges in Kentucky, Tennessee, and Louisiana under the program set up by the Division to provide visits and lectures to small colleges in the South.

D. E. Foard spoke at Western Kentucky State College, Bowling Green, Kentucky, on "New approaches to plant morphogenesis." W. E. Gude talked on "Biological effects of radiation" at East Tennessee State University. John Jagger visited Centenary College of Louisiana, Shreveport; Louisiana Polytechnic Institute, Ruston; and Northwestern State College of Louisiana, Natchitoches. His topics were "Light and life" at all three colleges and "How does uv radiation cause the mutation and death of a cell?" at the last two.

Courses. – The following lectures were given as part of the Visiting Scientist Program of the Tennessee Academy of Science:

W. D. Gude, "Biological effects of radiation on mammals," at Science Hill High School, Johnson City (four lectures) and at Greeneville High School, and "The use of autoradiography as a tool in research," at Greeneville High School.

John Jagger, "Cells, viruses, and DNA" and "Is there life on other worlds?" at Everett High School, Maryville.

An MEND (Medical Education for National Defense) lecture on "Secondary disease after bone marrow transplantation" was presented by C. C Congdon at the University of Oklahoma Medical Center, Oklahoma City.

As part of the ORNL-ORINS Ten-Week Course in Health Physics offered to graduate engineers, T. T. Odell, Jr., gave the following lectures: "Review of basic biology II. Gross anatomy and physiology" and "Acute injury and recovery in mammals after exposure to alpha, beta, gamma, or neutron radiation."

R. C. von Borstel presented three lectures in a course at the University of Florida Department of Entomology, Gainesville: "Kinetics of radiation effects on cells," "Dominant lethality and cell killing by radiation," and "Stage sensitivity and species response to radiation in different insects."

A. C. Upton presented a talk on "Radiation effects" in a course at the University of Pittsburgh School of Medicine. He also lectured on "Radiation hazards to man" at the Navy Nuclear Science Seminar held in Oak Ridge in December.

L. H. Smith and A. C. Upton spoke on "Radioprotective and recovery measures" and "Radiation and carcinogenesis," respectively, as part of a course in radiation pathology at the Armed Forces Institute of Pathology, Washington. M. L. Simmons attended a pathology course on Laboratory Animal Diseases at the Institute.

DIVISION MEMBERS ON LEAVES OF ABSENCE

Four staff members are away from the Division temporarily.

J. S. Kirby-Smith, Biophysics Group, has been on leave of absence and associated with the Division of Biology and Medicine of the Atomic Energy Commission, Washington, D.C., since January 1963.

D. R. Krieg of the Phage Genetics Group is on leave of absence with the Department of Chemistry, Stanford University, Palo Alto, California. His stay there is supported jointly by Oak Ridge National Laboratory and a special United States Public Health Service fellowship.

R. A. Wallace is spending ten months in Ottawa, Canada, at the National Research Council's Division of Applied Biology on collaborative work between that organization and the Biology Division.

Audrey N. Best, Enzymology Group, is attending the University of Georgia, Athens, as a candidate for the Ph.D. degree.

1. Cytology and Genetics

R. F. Kimball

Effects of Radiation on Paramecium (1.1–1.6)

R. F. Kimball
Satomi Igarashi[a]
D. C. Shepard[b]
G. L. Whitson[c]
E. G. Bailiff
H. Marine Scandlyn
Stella W. Perdue
Carrie L. Wells

Phage Genetics (1.7–1.11)

D. R. Krieg[d]
Jane K. Setlow
C. M. Steinberg
Amir Muhammed[a]
M. E. Boling
Ursula B. Steinberg[e]

Chromosome Cytology (1.12–1.16)

Sheldon Wolff
Sandra L. Bell[e]
K. M. Jakob[a]
J. E. Trosko[f]
H. E. Luippold
J. A. Heddle[a,g]

Fungal Genetics (1.17–1.18)

F. J. de Serres[h]
W. E. Barnett
H. E. Brockman
Anita M. Meley
Ida C. Miller
Carol A. Ortel
C. Jean Painter
Arlee P. Teasley
H. E. Kølmark[a]

Mammalian Genetic Cytology (1.18–1.19)

E. H. Y. Chu[h]
Tien-Ding Chang[a,i]
Lana T. Covington
Nancy H. Robinson
Patricia G. Lankford
Barbara A. Barbee[j]
Charlotte M. Boone[k]

Mammalian Biochemical Genetics (1.20–1.22)

R. A. Popp
Diana M. Popp
Judith G. Heddle

Cooperative NIH-AEC Chemical Co-Carcinogenesis Program (1.3 and 1.17)

[a]Visiting investigator from abroad.
[b]National Science Foundation Fellow.
[c]USPHS Fellow.
[d]Leave of absence, Stanford University.
[e]Consultant.
[f]Research associate.
[g]USPHS trainee.
[h]Dual assignment.
[i]Damon Runyon Memorial Fund.
[j]Temporary summer employee.
[k]Student trainee.

1.1 DISTRIBUTION OF X-RAY- AND ULTRAVIOLET-INDUCED MUTATIONS TO THE CHROMOSOME STRANDS IN *PARAMECIUM*

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

Introduction. – In the previous semiannual report,[1] data on distribution of x-ray and ultraviolet mutations to the products of the first three post-irradiation divisions were reported. With either radiation, only two distributions of mutations were found with significantly greater frequency in the irradiated than the controls: (1) all progeny from one product of the first division but not the other mutant (half mutations) and (2) all progeny mutant (whole mutations). These classes would be expected if one or both of the two pre-existing strands

that are present at the time (preduplication interphase) of irradiation were affected. The classes that would be expected if only the new strands were affected, quarter and double-quarter mutations, were no more frequent than in the unirradiated control. The data suggested that ultraviolet produced relatively more half mutations than x rays, but this conclusion was subject to doubt because the ultraviolet dose was so high that multiple mutations were produced and the relative frequencies had to be inferred indirectly by a formula allowing for the obscuring of half mutations by wholes. Moreover these multiple mutations might have hidden quarter and double-quarter mutations. Therefore the ultraviolet experiment was repeated with a lower dose. Additional x-ray data have been obtained also.

Methods. – The methods were essentially the same as given in the previous report. Paramecia were irradiated in preduplication interphase, the products of division were put into separate containers after each of the next three divisions, the eight products so obtained were allowed to multiply to form small cultures, autogamy was induced, and the presence of mutations was inferred from the presence of slowly growing or inviable autogamous progeny. Sisters of the irradiated cells were sent through this same procedure to serve as an unirradiated control. The paramecia were irradiated with about 500 r of 250 kvp x rays or about 160 ergs/mm^2 incident intensity of 2537 A ultraviolet.

Results and Discussion. – The numbers of mutations, after subtraction of the control values, are shown in Table 1.1.1 for the complete x-ray and the new ultraviolet data. The only classes of mutations appearing with a significantly higher frequency in the irradiated than in the controls were wholes and halves. Quarters, double-quarters, eighths, etc., were rare and no more frequent than in the control. The ultraviolet data, unlike the earlier ones at higher dose, do not appear to differ from the x-ray data in the relative frequency of wholes and halves, suggesting that the apparently greater relative frequency of halves in the high-dose experiment was an artifact of the method of interpreting the data. The laboriousness of the experiments has kept the numbers small, however; more ultraviolet data will be required to be certain that there is no great difference between the two radiations. Nevertheless, the evidence is clear that ultraviolet produces an appreciable frequency of whole mutations, implying that many of the ultraviolet premutational events affect both of the conserved chromosomal strands that are present in G1. The evidence is also clear in showing that most if not all of the ultraviolet mutations affect the pre-existing as well as the new strands; otherwise an appreciable frequency of quarter or double-quarter mutations would have been produced.

Table 1.1.1. Number of Induced Mutations

Class	X Rays		Ultraviolet	
	Number	Percent	Number	Percent
Whole mutations	15	10.5	6	10.9
Half mutations	4	2.8	2	3.6
No mutations	124	86.7	47	85.5

[1] R. F. Kimball *et al.*, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 29–30.

1.2 EVALUATION OF AUTORADIOGRAPHIC METHODS FOR STUDYING OVERALL CELL GROWTH

R. F. Kimball Stella W. Perdue
H. Marine Scandlyn

Introduction. – Most cell lines have a characteristic cell size to which they return even when the size has been drastically altered in some way. The size of the cell at division, a useful standard point, is determined by two measurable parameters, the rate of growth during the cell cycle and the duration of the cycle. Measurement of these parameters is essential for careful analysis of the maintenance and restoration of cell size and of the occasional failures of restoration. Earlier we measured cell dry weight at various stages during the cycle by a microinterferometric procedure and drew certain conclusions about the control of cell growth.[1] Autoradiographic methods are also potentially of value for measuring cell growth and are technically simpler. The present report concerns attempts to apply two autoradiographic procedures

to this problem and to draw some conclusions about the general suitability of such procedures compared to the microinterferometric one.

Methods. – Paramecia at known stages during the cell cycle were obtained by picking out dividing specimens and killing samples at various times after division. Autoradiographs were prepared, and the grains per unit area of cytoplasm were counted. These counts are proportional to the amount of labeled material per unit mass in thick specimens, such as dried paramecia.

Two labeling procedures were used: (1) pulse labeling with killing immediately after the pulse and (2) dilution of previously incorporated conserved label[2] by increase in unlabeled cytoplasmic mass. The pulse-labeling experiments measure the rate of incorporation of a labeled compound, tritiated leucine in this case, per unit cytoplasmic mass. The dilution experiments measure the increase in unlabeled cytoplasm relative to the mass at the beginning of the cycle. To do this efficiently, the ratio was taken between the grain counts over a cell dried immediately after division and over its sister dried some time later.

Results and Discussion. – The pulse-label experiments indicated a nearly constant rate of incorporation per unit mass throughout the cell cycle. This is the result expected with the approximately exponential increase in cell mass found in our microinterferometric work.[1] It differs from a previous pulse-labeling experiment,[3] which showed a maximum rate of incorporation per unit mass midway in the cycle. The two experiments differed in the length of the pulse and possibly in some uncontrolled variables. The pulse-labeling experiment is potentially able to yield valuable information because of its high sensitivity to small changes in rate, but this very sensitivity may make it difficult to control and so not very suitable for large-scale measurements of cell growth.

The second procedure, dilution of a conserved label, proved to be subject to a greater variance than we had hoped, although repeats with different labels were in good agreement with one another, considering the high variance. The results confirmed the earlier evidence[2] for conservation of label from amino acids and from pyrimidine nucleosides. Despite quite large numbers, however, a statistically valid distinction between a linear and an exponential increase in mass was not achieved. This distinction was made easily by the much less variable microinterferometric measurements of our earlier work.[1] In any case the dilution experiment is restricted to those metabolic conditions in which label is completely conserved. This restriction and the high variance make it unlikely that it can be developed into an adequate method for studying cell growth.

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

[2] R. F. Kimball, Stella W. Perdue, and H. Marine Scandlyn, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 29.

[3] B. A. Lowery and R. F. Kimball, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 32.

1.3 STUDIES ON CHEMICAL MUTAGENESIS IN *PARAMECIUM*[1]

R. F. Kimball H. Marine Scandlyn
E. G. Bailiff

Introduction. – In the past we have used *Paramecium aurelia* to study (1) the relative efficiency of various radiations, with or without auxiliary treatments, for mutagenesis and for inhibition of growth and cell division, and (2) the ability of the cell to recover from or repair initial damage, premutational or other. We would like to extend these studies to various chemical agents, especially chemical mutagens; and the present report is a beginning in this direction.

Results and Discussion. – Some information is already available for the alkylating agent, nitrogen mustard, suggesting but not proving the possibility of posttreatment repair of potential premutational damage.[2] We have now obtained definite proof that such repair is possible by use of posttreatment with streptomycin, a procedure that had already proved quite effective with various radiations.[3] It is quite possible that streptomycin acts, just as it does for the radiations, by increasing the time available for repair.

Nitrogen mustard, like ultraviolet, has one disadvantage for use in such repair studies. Mutagenic doses considerably delay division and, presumably, DNA synthesis thus complicating the interpretation of results with auxiliary treatments. We have now found that triethylene melamine has almost no

effect on growth and division in *Paramecium* at doses that are highly mutagenic. In this respect, it resembles ionizing radiation rather than ultraviolet and nitrogen mustard.

[1]NIH-AEC Chemical Co-Carcinogenesis Program.

[2]R. F. Kimball, Stella W. Perdue, and H. Marine Scandlyn, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, pp. 31–33.

[3]R. F. Kimball, Nenita Gaither, and Stella M. Wilson, *Radiation Res.* **10**, 490 (1959).

1.4 INDUCED TEMPERATURE-SENSITIVE MUTATIONS IN *PARAMECIUM AURELIA*

Satomi Igarashi R. F. Kimball

Introduction. – Previous work on mutation induction in *Paramecium aurelia* has been done with lethal and slow-growth mutation,[1] a class that might include mostly changes, such as small deficiencies, that cannot code for particular proteins at all. It seemed desirable to extend the work to mutations of a class that is more likely to include changes, such as base pair alterations, that could code for an altered protein. Such a class might be temperature-sensitive mutations that are lethal at high temperature but viable at low. Such conditional lethals have the added advantage that they can be kept in stock culture and analyzed genetically, something which is difficult to do with the mutations previously used. The present report shows that persistent temperature-sensitive autogamous segregants do occur in x-irradiated material and takes up some of the problems in detecting them.

Methods and Results. – *Temperature Sensitivity of Normal Paramecia.* – *Paramecium aurelia*, syngen 4, stock 51, mating type VII, was studied for the temperature sensitivity. Dividing animals were picked up and one sister cell was incubated at 27°C for three days, while the other was kept at the higher temperatures. It became clear that 37.5°C is the critical temperature in this stock. Therefore the slightly lower temperature, 36.5°C, was adopted as the temperature at which the sensitivity was to be tested. At this temperature, normal paramecia of this stock survive. No especially temperature-sensitive period was found in the division cycle, but normal exautogamous paramecia are temperature sensitive for at least 7 hr after enrichment.

X-Ray-Induced Temperature Sensitive Mutants. – The paramecia were irradiated by 2.5 kr with 250 kvp x rays at about 1.7 kr/min. Paramecia were irradiated at various phases of the cell cycle and isolated immediately after treatment. After four days of culture, autogamy was induced and exautogamous specimens were isolated into fresh medium. Dividing animals were picked up after 20 hr of culture for supplying one sister cell as a control and the other sister as an experimental, which was incubated at 36.5°C. This procedure avoids the temperature-sensitive period of the wild type itself and provides a check for temperature-independent death or slow growth of the clone. After three days of culture, the growth of the sister cultures was compared. The clones which could grow at 27°C and could not at 36.5°C were potentially temperature-sensitive mutants and were kept in the tube culture. Some, though not all, of these clones remained temperature sensitive through several successive autogamies. Estimation of the frequency is as yet uncertain, but there were at least several percent at 2.5 kr, perhaps higher.

Induction Rate and Relating Factors. – The induction rate at various phases of the cell cycle was higher at zero time than at 2 hr after fission. This is the opposite of what is found with lethal or slow growth mutations,[1] but the evidence is still not certain.

A number of uncontrolled factors seem to cause very large variations of the induction rate that are not understood. For instance, the difference in the time between irradiation and the first autogamy has a marked effect on the induction rate.

In any case, as the cross-breeding test with the complementary mating type has not yet been done, it is uncertain whether the system of temperature sensitivity is Mendelian or not. The fact that some clones still show the temperature sensitivity after several autogamies suggests that this trait is inherited genically, but cytoplasmic inheritance cannot be eliminated without breeding tests.

[1]R. F. Kimball, pp. 167–78 *in Repair from Genetic Radiation Damage and Differential Radiosensitivity in Germ Cells* (ed. by F. H. Sobels), Pergamon, New York, 1963.

1.5 DNA CONTENT OF ULTRAVIOLET-IRRADIATED *TETRAHYMENA*

D. C. Shepard

Introduction. – A number of the ciliate protozoa have been shown to exhibit division delays over several generations following ultraviolet irradiation.[1] Cells during the initial postirradiation cell generations appear larger than normal, whereas during the later delayed generations the cells are smaller than normal. Because the synthesis of DNA is particularly sensitive to ultraviolet radiation and because irradiation appears to interfere with the "normal" doubling of cell constituents between divisions, the DNA content of *Tetrahymena* has been determined for several cell generations after ultraviolet irradiation.

Materials and Methods. – *Tetrahymena pyriformis* strain W (an amicronucleate strain) was cultured axenically in 2% proteose peptone at (25 ± 0.5)°C. From a log phase culture, dividing cells were isolated individually, and within 30 min of division one sister of each pair was air dried in a recorded position on a "subbed" microscope slide. The remaining sisters were irradiated at a known time during the cell generation cycle, some early in the cycle, some late. Immediately following the next three divisions, one sister was air dried in another recorded position. The cells were then stained for DNA by the Feulgen procedure, and the relative amount of Feulgen positive material in each cell was determined by two-wavelength microspectrophotometry. The dilution of incorporated tritiated thymidine was followed through several generations by making grain counts on autoradiographs over a constant area of the macronucleus of Feulgen stained cells. In this case, tetrahymenas were cultured for 4 hr in medium to which 1 μc/ml of tritiated thymidine was added. The cells were washed free of isotope by centrifugation before dividing cells were selected for the radiation experiment.

Results. – The DNA content for unirradiated and irradiated, recently divided tetrahymenas over three generations has been obtained. The results for the irradiated tetrahymenas, irrespective of the time during the cell generation when irradiated, are shown in Table 1.5.1. The DNA content of unirradiated tetrahymenas showed no significant differences over the three generations, and the generation time for unirradiated cells was about 3.5 hr. During the first postirradiation cell generation, there is an increase in DNA content, which is evident for one more generation. However, the excess DNA is insufficient to provide for the DNA content after the second and third generations without further DNA synthesis during these later divisions. The increased DNA content is apparent even when tetrahymenas are irradiated during the last hour of the cell cycle, a time when similarly grown tetrahymenas show no uptake of tritiated thymidine and no measurable increase of Feulgen positive material.

Table 1.5.1. Feulgen Positive Material Content of Recently Divided, Irradiated *Tetrahymena*

Division	Average Generation Time (hr)	Number of Cells Measured	Relative DNA Content (Mean ± Standard Error)
0[a]		92	90.0 ± 2.41
1	12.7	44	142.1 ± 7.64
2	6.4	62	106.0 ± 3.65
3	5.8	53	89.7 ± 3.55

[a]Sister cell dried before irradiation.

The concentration of previously incorporated thymidine is halved at each division of unirradiated tetrahymenas. In irradiated tetrahymenas the concentration of isotope is decreased by more than half during the first generation and less than half during the second and third generations, confirming the DNA content measurements. There is an indication that there may be a greater dilution of isotope during the first and second generations than can be explained on the basis of the DNA contents, possibly indicating that some macronuclear component other than DNA is also changing disproportionately.

[1]R. F. Kimball, *Ann. Rev. Microbiol.* 11, 199 (1957).

1.6 SOME EVIDENCE FOR THE ROLE OF RNA SYNTHESIS IN REGENERATION IN *STENTOR COERULEUS*

G. L. Whitson

Introduction. – It is well known that when cells of the ciliated protozoan *Stentor coeruleus* are cut

in half they will regenerate their missing parts.[1] However, very little is known concerning RNA synthesis during regeneration.[2] Therefore, as a first approach to this problem, cytochemical studies were begun by using ribonuclease, actinomycin-D, and autoradiography.

Methods. – Cells of *S. coeruleus* were cut in half, and isolated tail pieces were used in experiments on regeneration. In the initial experiments, isolated tail pieces were collected and placed in ordinary culture fluid to time the period for normal regeneration. In other experiments, tail pieces were exposed continuously to known concentrations of either ribonuclease or actinomycin-D in an attempt to inhibit regeneration. Additional experiments included autoradiographic studies on the uptake of H^3-cytidine during regeneration.

Results. – Regeneration in isolated tail pieces from well-fed cells occurs normally in 6 to 8 hr in ordinary culture medium. Continuous exposure to 1000 μg/ml of ribonuclease completely inhibits regeneration, and exposure to 125 μg/ml has a detectable effect. Continuous exposure to 250 μg/ml of actinomycin-D completely inhibits regeneration, and exposure to 50 μg/ml has a detectable effect. Even at the highest concentrations of both substances the pieces survive for many hours.

For autoradiography, uncut cells and tail pieces from cut cells were put into H^3-cytidine for the full period of regeneration. Autoradiographs of sections showed both nuclear and cytoplasmic incorporation, and the label was removable by pretreatment of the sections by ribonuclease. Even though the cells were starved, the uncut controls incorporated the riboside, making it impossible to detect any special RNA synthesis in the regenerates. It was shown, however, that concentrations of actinomycin-D that markedly inhibit regeneration also inhibit RNA synthesis.

Discussion. – Studies on regeneration and nucleocytoplasmic interactions in *Stentor*[1,2] have shown that the nucleus plays an essential role in the formation of new oral structures. However, the exact nature of this nuclear contribution to regeneration is not known. In view of the present results, it appears that both the presence of RNA and nuclear RNA synthesis are necessary for regeneration.

[1]V. Tartar, *The Biology of Stentor*, Pergamon New York, 1961.

[2]V. Tartar, *J. Protozool.* **10**, 445 (1963).

1.7 MUTAGEN-DIRECTED NUCLEOTIDE REPLACEMENTS IN DNA

D. R. Krieg

Research has been continued to identify directly the types of nucleotide composition changes that occur during DNA duplication after treatment by a mutagen. A previous report[1] described a preliminary experiment of this type, in which a homopolymer of deoxycytidine, after being treated with the mutagen hydroxylamine, was found to direct the polymerization of a measurable amount of deoxyadenine together with deoxyguanine, the normally polymerized complement of deoxycytidine. The possible uniqueness and further characterization of this apparent mutagen-directed nucleotide "incorporation error" is under investigation. Recent efforts have been directed toward gaining increased familiarity with the in vitro DNA polymerase system employed, the development and refinement of techniques, and the preparation of sufficient quantities of materials for a more detailed investigation.

[1]D. R. Krieg, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 33.

1.8 EVIDENCE FOR A SECOND PHOTOREACTIVABLE LESION IN DNA

Jane K. Setlow M. E. Boling

Introduction. – It is possible to account for all or almost all of the biological effects of in vitro photoreactivation of transforming DNA in terms of splitting of ultraviolet-induced thymine dimers by light in the presence of yeast extract.[1] However, Rupert has found that ultraviolet-irradiated poly deoxy GC not only competes with ultraviolet-irradiated transforming DNA for the active agent in yeast extract, but the ability of this substance to compete may be eliminated by preillumination with yeast extract.[2] These two sets of observations may be reconciled with the hypothesis that there are two types of lesions which are reversed by the photoreactivating agent in yeast extract, one of

which is the thymine dimer and the other is in G or C, but the biological activity of the transforming activity is not affected by the second type of lesion. Two types of experiments have been performed to test this hypothesis.

Results and Discussion. – DNA has been treated with various large doses of 2378-A radiation, so that the measured number of thymine dimers was the same in all the samples. If the kinetics of photoreactivation are only the result of splitting of thymine dimers, then the rates of photoreactivation and dimer splitting, as well as the competing ability, of these samples should be the same after the different doses. However, the rates of photoreactivation and dimer splitting decreased with increasing dose, whereas the competing ability increased, suggesting that a lesion other than the thymine dimer increases with increasing dose and competes with the thymine dimer for the active agent in the yeast extract.

A strain of *Escherichia coli* has been found in which there is no photoreactivation of colony formation,[3] but there is photoreactivation of certain mutations.[4] Incubation of irradiated transforming DNA with an extract from this mutant in the light does not increase the transforming ability (unlike extracts made from the wild type), but it does alter the kinetics of subsequent photoreactivation with the yeast extract. At low intensities of photoreactivating illumination with the yeast extract, there is a pronounced lag in the reaction. This lag is eliminated by preincubation of the irradiated DNA with the *E. coli* extract in the light (but not in the dark), suggesting that whereas the mutant lacks the ability to split thymine dimers, it can reverse a second ultraviolet lesion which normally competes with the thymine dimer for the active agent of the yeast extract. We may assume that the second lesion does not affect transforming ability, and thus there is no change in this property of the DNA during the period when the second lesion is successfully competing with the thymine dimer, so that what is observed is a lag in photoreactivation and a lag in thymine dimer splitting.

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

[2] C. S. Rupert, *J. Cell. Comp. Physiol.* **58**(1), 57 (1961).

[3] W. Harm and B. Hillebrandt, *Photochem. Photobiol.* 1, 271 (1962).

[4] E. Witkin *et al.*, *Proc. Natl. Acad. Sci. U.S.* **50**, 1055 (1963).

1.9 ULTRAVIOLET RESISTANCE OF *MICROCOCCUS RADIODURANS*

Jane K. Setlow D. E. Duggan[1]

Introduction. – *Micrococcus radiodurans* is an organism which is exceptionally resistant to ultraviolet radiation[2] as well as to ionizing radiation.[3] In order to determine whether the ultraviolet resistance is due to shielding of the DNA by other absorbing compounds, innate resistance of the DNA, or to an extremely effective repair mechanism, thymine dimer formation has been measured as a function of ultraviolet dose to intact cells. This reaction has been shown to be responsible for most of the biological effect of ultraviolet radiation on DNA.[4]

Results and Discussion. – The DNA of the micrococcus is not exceptionally resistant to thymine dimerization. For example, a dose of 3000 ergs/mm^2 at 2652 A, which produces *no* loss of viability, results in 1% of the total thymine appearing as dimer. The DNA of *Escherichia coli* is about three times less resistant than that of *M. radiodurans* to thymine dimerization, although this by no means accounts for the much higher resistance of *M. radiodurans* to inactivation. The difference in resistance to dimerization may be due partly to the difference in base ratio and partly to the difference in absorption per cell. We have found that *M. radiodurans* has a (G + C)/(A + T) ratio of 1.6 as compared with 1.0 for *E. coli*, so that whereas about 6.2% of the bases in *E. coli* appear in TT sequences, we would expect the corresponding figure for *M. radiodurans* to be 3.7%. The absorption per cell at 2652 A, calculated from the measured absorption of a cell suspension in which the number of cells was counted microscopically, is 0.27 for *M. radiodurans*, 0.68 for *E. coli*.

The data suggest that the micrococcus has a very effective way of repairing ultraviolet lesions. The kinetics of postirradiation DNA synthesis suggest what the time course of such repair might be. The rate of DNA synthesis was measured by determining the incorporation of labeled thymidine into cells. We observed an ultraviolet-induced DNA synthesis delay similar to that previously seen in *E. coli*,[5] except that the doses used for *M. radiodurans* are more than 20 times larger, suggesting a much more rapid and efficient repair mechanism than in *E. coli*.

[1] Radiation Microbiology Group.

[2]D. E. Duggan *et al.*, *Food Res.* **24**, 376 (1959).

[3]A. W. Anderson *et al.*, *Food Technol.* **10**, 575 (1956).

[4]R. B. Setlow and Jane K. Setlow, *Proc. Natl. Acad. Sci. U.S.* **48**, 1250 (1962).

[5]R. B. Setlow *et al.*, *Science* **142**, 1464 (1963).

1.10 ACTION SPECTRUM FOR KILLING AND DNA SYNTHESIS DELAY IN *MICROCOCCUS RADIODURANS*

Jane K. Setlow M. E. Boling

Introduction. – Since *Micrococcus radiodurans* apparently has a very effective repair mechanism for ultraviolet lesions, it is of interest to examine the shape of the action spectrum for killing, in the hope that it might suggest what are or are not the lethal lesions. The wavelength dependence for ultraviolet-induced DNA synthesis delay could also suggest the type of lesion whose repair causes the delay.

Results and Discussion. – The survival curve of this organism has an initial flat portion and final exponential portion. The action spectrum for killing was obtained in two ways: (1) from the slopes of the exponential portions and (2) from the intercepts of the exponential portions, which represent the lowest doses at which the cells begin to be inactivated. The action spectra are approximately the same, indicating that the shape of the survival curve is independent of wavelength. The action spectra differ in two respects from the usual action spectra for inactivation of bacteria, bacteriophage, or transforming DNA: (1) 2805 A is only a little less effective than 2652 A in killing the micrococcus and (2) the sensitivity to the least effective wavelength (about 2450 A) is only about one-half that of the most effective wavelength (about 2700 A), whereas one-third to one-fourth is the usual figure. Except in the 2805 A region, the spectra resemble cytosine deoxyriboside absorption, suggesting that the lethal lesions could be damage in this substance or in protein.

The amount of ultraviolet-induced delay in DNA synthesis is a linear function of dose. The sensitivity varies considerably with the particular stage of logarithmic growth, so that it was necessary to irradiate cells at exactly the same stage in order to compare the delaying effect of one wavelength to that of another. The action spectrum obtained is almost the same as that for killing. Since we have found that the relative efficiencies of 2378, 2652, and 2805 A for dimerization of thymine in the DNA when the micrococci are irradiated whole are *not* like the action spectra for killing and DNA synthesis delay in the micrococcus, but resemble the usual action spectra for killing, we conclude that the thymine dimer is *not* the lethal or DNA-synthesis delaying event in *M. radiodurans*. This suggests that this organism has a particularly rapid and effective repair system for the thymine dimer.

1.11 PURIFICATION OF THE PHOTOREACTIVATING ENZYME FROM YEAST

Amir Muhammed

Introduction. – Rupert[1] has described a photoreactivating (PR) enzyme in yeast which is capable of partially repairing ultraviolet damage in transforming DNA. Most of the earlier work reported in the literature has been done with preparations only about fivefold purified from the crude yeast extract. The nature of the prosthetic group of the PR enzyme which presumably absorbs the photons as a first step in photoreactivation is not known. It is essential to have a highly purified enzyme preparation in order to determine the nature of the chromophore in the PR system.

Experimental Results. – The enzyme has been purified approximately four hundred fold with ammonium sulfate, DEAE, and Sephadex G-200. The fact that the enzyme forms a stable complex with ultraviolet-irradiated DNA in the dark has been utilized to separate the complex of enzyme and ultraviolet-irradiated DNA from the bulk of the inactive proteins in the yeast extract by Sephadex G-200 filtration. Efforts are being made to recover the active enzyme from the complex by illuminating it.

Three main results have been obtained from the fractionation studies.

1. Enzyme activity depends on the concentration of phosphate buffer; above 0.02 *M* there is inhibition, and at 0.2 *M* or higher there is no detectable activity. Presumably high phosphate concentration prevents complex formation.

2. When the enzyme is eluted from a DEAE cellulose column with a phosphate gradient, two peaks are obtained in a plot of specific activity vs effluent volume. It is possible that the two peaks represent two different enzymes responsible for PR, or two forms of the same enzyme with different charge.

3. Loss of enzyme activity on purification has been found to be due to the extreme sensitivity of the enzyme to heavy-metal ions. There is almost complete inhibition of enzyme activity by Hg^{2+} (5×10^{-6} *M*), Ag^{+} (5×10^{-6} *M*), Cu^{2+} (1×10^{-5} *M*), and Zn^{2+} (1×10^{-4} *M*). The problem has been largely solved by adding EDTA routinely to all solutions of the enzyme.

[1]C. S. Rupert, *J. Gen. Physiol.* **43**, 573 (1960).

1.12 EFFECT OF FAR-RED RADIATION ON MITOSIS

Sheldon Wolff H. E. Luippold

Introduction. – Far-red radiation when given in combination with x rays results in an increased yield of chromatid aberrations. Experiments with *Vicia faba* root tips have shown that this is most likely caused by an inhibition of the rate at which cells proceed through the mitotic cycle.[1] Thus the curve for aberrations found at given times after irradiation is shifted if far-red radiation is administered. The apparent synergism is, therefore, not caused by an effect on chromosome breakage or rejoining but is simply a reflection of the fact that cells that are in different parts of the cell cycle show a differential sensitivity to x rays and that cells that were in the same part of the cycle at the time of far-red treatment do not arrive at metaphase at the same time as cells exposed to x rays alone.

Results. – In order to test directly the effect of far-red radiation on mitosis, lateral roots of *Vicia* were treated with tritiated thymidine for 15 min to label cells in the DNA synthetic portion of the cell cycle (S period). Roots were then exposed to 150 r of 250 kvp x rays followed by 3 hr of exposure to light from fluorescent lights or 3 hr of exposure to far-red radiation (*ca.* 800 mμ, 37,000 μw mm^{-1} sec^{-1}). Other roots received similar treatment but were not exposed to x rays. At various times after treatment roots were fixed and the percentage of metaphases that came from S (i.e., were labeled) was determined. The results are presented in Fig. 1.12.1.

Discussion. – Figure 1.12.1*a* shows that far-red radiation alone causes a mitotic delay so that labeled cells from the S period reach metaphase at a later time. X radiation alone causes a similar delay in that the peak of labeled metaphase cells occurs later than it does in unirradiated roots (Fig. 1.12.1*b*). In addition the peak is broadened. The delay in mitosis caused by far-red radiation is still evident over and above the x-ray-induced delay.

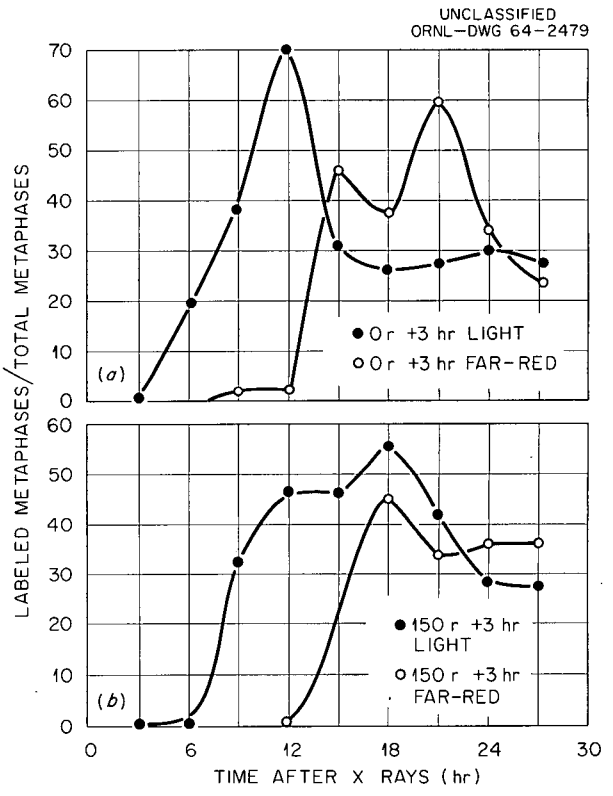

Fig. 1.12.1. Effect of Far-Red Radiation on Time of Appearance of S Cells at Metaphase.

Far-red radiation has, thus, been found to induce a delay in mitosis so that it takes longer for far-red-treated cells to proceed from interphase to metaphase. Such delays are consistent with the shift in time of appearance of aberrant cells at metaphase.

[1]Sheldon Wolff and H. E. Luippold, pp. 457–60 in *Proc. 3rd Intern. Congr. Photobiol.* (ed. by B. Christensen and B. Buchman), Elsevier, Amsterdam, 1960.

1.13 KINETICS OF NEUTRON- AND X-RAY-INDUCED CHROMOSOMAL ABERRATIONS IN BARLEY

J. A. Heddle

Introduction. – Experiments performed on *Vicia faba* have shown that there is no interaction between x-ray- and neutron-induced chromosome breaks.[1] In addition, independently induced neutron breaks do not interact among themselves as shown by a linear dose response curve, while x-ray breaks do interact resulting in an exponential dose curve. The lack of interaction of x-ray- and neutron-induced breaks has been attributed to the spatial relations of the two strands that must be broken to produce a two-break aberration. Recently, however, Natarajan and Narayanan[2] have reported that in barley seeds there is complete interaction among x-ray- and neutron-induced chromosome breaks. From this it would be predicted that in barley there would be an exponential increase in the frequency of chromosome aberrations with neutron dose and a fractionation effect.

Results. – Barley seeds of two moisture contents, 4 and 10%, were irradiated with various doses of 14.1-Mev neutrons and the first mitotic metaphases in the root tips scored for aberrations. In seeds of either moisture content, the frequency of two-break aberrations (dicentrics and rings) increases in direct proportion to dose (Fig. 1.13.1). This indicates that in barley, as in *Vicia*, both chromosome strands that form the aberration are broken simultaneously, rather than independently as predicted from Natarajan's data. Similarly when a dose fractionation experiment was performed, no fractionation effect was found (Table 1.13.1).

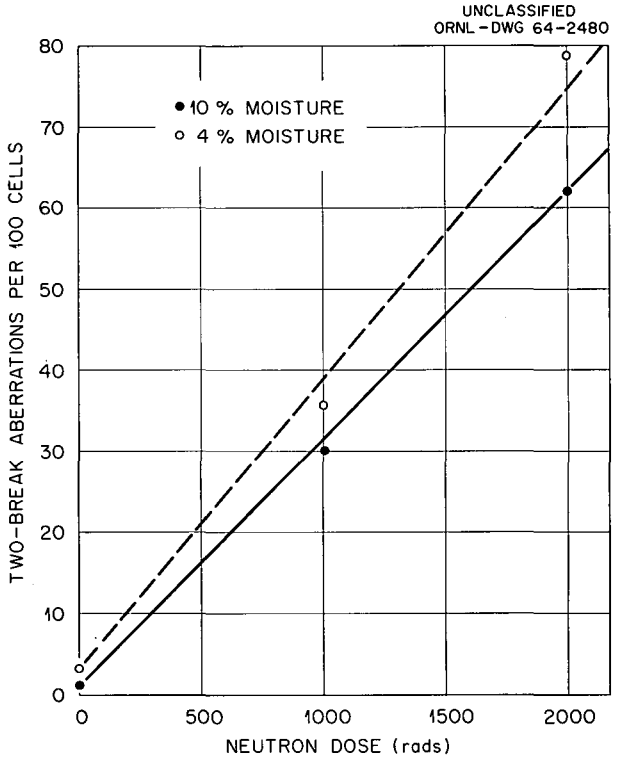

Fig. 1.13.1. Kinetics of Two-Break Aberrations in Barley After Neutron Irradiation

Table 1.13.1. Results of a Neutron Fractionation Experiment on Barley Seeds of Two Moisture Contents

	Number of Cells	Two-Break Aberrations[a] (%)	Terminals (%)
4% moisture content			
Controls	300	3.0	3.0
2000 rads continuous	275	78.9[b]	34.6
2000 rads fractionated	249	67.0[b]	25.5
10% moisture content			
Controls	300	1.0	2.3
2000 rads continuous	243	62.0[b]	28.4
2000 rads fractionated	300	53.6[b]	19.3

[a]Two break, X = dicentrics + rings + 2 × tricentrics.
[b]P value, 0.1 to 0.2 (from t test).

Discussion. – The lack of interaction of x-ray- and neutron-induced breaks has been attributed to the fact that in *Vicia* the rejoining distance over which broken ends can move to rejoin with one another is small. A short rejoining distance is also the reason for linear dose response curve of two-break aberrations with neutrons. If x-ray and neutron breaks interact, we might expect that the rejoining distance is larger in barley and that this would lead to a nonlinear neutron response curve. The present experiments show that if the rejoining distance does increase in barley, the increase is insufficient to cause a deviation from linear kinetics. Experiments are now in progress to determine whether or not x-ray-induced breaks will interact with neutron-induced breaks in barley and also to estimate the rejoining distance.

[1]Sheldon Wolff *et al.*, *J. Biophys. Biochem. Cytol.* **4**, 365–72 (1958).

[2]A. T. Natarajan and K. C. Narayanan, pp. 413–26 in *Repair from Genetic Radiation Damage* (ed. by F. H. Sobels), Macmillan, New York, 1963.

1.14 STUDIES OF THE MECHANISM OF THE EFFECT OF 5-FLUORODEOXYURIDINE ON CHROMOSOMES

Sandra L. Bell Sheldon Wolff

Introduction. – 5-Fluorodeoxyuridine (FUdR) has been shown to break chromosomes in *Vicia faba*. Since FUdR had previously been found to inhibit DNA synthesis in virus-infected *Escherichia coli*,[1] and because the time required for the appearance of breaks in *V. faba* was equal to the length of the G_2 period in untreated cells, it was originally postulated that the chromosomes were broken in the latter portion of the DNA synthesis (S) period of the cell cycle.[2] It has recently been shown, however, that the chromosomes can be broken in all parts of the cell cycle and not only in the S period.[3] When *Vicia faba* root tips are treated with equal amounts of FUdR and thymidine, there is no inhibition of DNA synthesis. In spite of this, chromosome aberrations are still produced. The chromosome breaking effect of FUdR is reversed only by the addition of greater amounts of thymidine, indicating that this effect is independent of any possible effect on DNA synthesis.

Since FUdR had never been shown directly to affect DNA synthesis in *Vicia faba*, or in any other higher plant, it was therefore decided to see if there indeed was an effect on DNA synthesis.

Results. – Experiments were carried out in which lateral roots were grown in a mixture of 10^{-5} *M* FUdR and 10^{-5} *M* uridine for 1 hr and then transferred to H^3-cytidine for 30 min. H^3-Cytidine was used because in bacteria thymidine can reverse the effect of FUdR on DNA synthesis. The roots were fixed in neutral formalin and autoradiographs prepared. Since cytidine is incorporated into both RNA and DNA, ribonuclease was used on the slides to remove the H^3-cytidine-labeled RNA. When grain counts on autoradiographs were made it was found that the FUdR-treated roots had fewer grains over the nuclei than the control (10^{-5} *M* uridine) roots. FUdR inhibited DNA synthesis. Since, however, there was some label in FUdR-treated nuclei, it is not certain that the FUdR had completely inhibited DNA synthesis. Experiments are now being carried out to see if this label is deoxyribonuclease-removable or if it is in ribonuclease-resistant RNA.

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

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

[3]Sandra L. Bell and Sheldon Wolff, to be published in *Proc. Natl. Acad. Sci. U.S.*

1.15 SOME EFFECTS OF POSTIRRADIATION CENTRIFUGATION ON CHROMOSOMAL ABERRATIONS IN *TRADESCANTIA* MICROSPORES

K. M. Jakob Sheldon Wolff

Introduction. – It is known that centrifugation (2300 × *g*) at room temperature immediately after x irradiation increases the number of chromosome exchanges in both *Tradescantia* microspores and first root tip divisions in soaked seeds of *Vicia faba*.[1] Preirradiation centrifugation on the other hand will reduce the number of exchanges. The

effect on x-ray-induced aberrations of centrifugation speed, time, and temperature has not yet been clarified. Since two-break aberrations are the result of both chromosome breakage and the subsequent rejoining of the broken ends into new configurations, any treatment that affects breakage, rejoining, or the number of sites where rejoining can occur[2] could affect the aberration yield. Experiments were performed to gain some information on the possible means by which centrifugation affects the yield of aberrations.

Results. – *Tradescantia* inflorescences were exposed to 200 r of x rays at 500 r/min at room temperature. The inflorescences were centrifuged immediately after irradiation; 4 days later when the first cell division occurred, microspores were analyzed for chromosome aberrations.

When postirradiation centrifugation was performed for 10 or 20 min at 2°C there was little or no increase in two-break aberrations at 2500, 5000, or 10,000 × *g*. Twenty minutes (but not 10 min) at 1000 × *g*, however, resulted in a decrease in two-break aberrations as compared to the noncentrifuged x-ray controls kept for the same time at 2°C. The relative drop in two-break aberrations was greater when compared to a noncentrifuged control kept at room temperature.

Discussion. – Postirradiation treatment with low temperatures will keep chromosome breaks open.[1] The increase in chromosome exchange brought about by postirradiation centrifugation at 2300 × *g* at room temperature has been interpreted to be a consequence of chromosome movement which reduces restitution and correspondingly increases the frequency of exchange.[1]

The present results suggest that both the temperature and the time of centrifugation influence rejoining of broken chromosomes. Several possible interpretations that are testable exist. For instance it might be that when centrifugation is performed at room temperature, the rejoining of broken ends takes place during centrifugation, whereas at 2°C, the rejoining process would be slowed down, and much or all of the rejoining would take place later. As there is a limit, even under low-temperature conditions, to the time during which chromosome ends may stay open without losing their ability to rejoin, a long cold treatment could result in more open ends that cannot rejoin. We have indications that there is a decrease in two-break aberrations and an increase in one-break ones with increasing time of postirradiation cold treatment.

Thus a part of the decrease in two-break aberrations following 20 min at 1000 × *g* might be explained as caused by an excess of nonrejoinable broken ends produced by the extended cold treatment during centrifugation. Another possibility is that with low centrifugal force, there is a packing effect on chromatin that changes the number of sites for chromosome exchanges. Such effects were noted in experiments in which the centrifugation preceded the radiation.[1]

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[1] Sheldon Wolff and R. C. von Borstel, *Proc. Natl. Acad. Sci. U.S.* **40**, 1138–41 (1954).

[2] Sheldon Wolff, *J. Theoret. Biol.* **3**, 304–14 (1962).

1.16 STUDIES ON THE ISOLATION OF FIXED METAPHASE CHROMOSOMES

J. E. Trosko Sheldon Wolff

Introduction. – Because of the lack of information on the dynamic relations of chromosomal components, radiation-induced genetic, cytological, and biochemical damages have not been correlated simultaneously. We, therefore, made attempts to isolate chromosomes so that questions concerning the roles the different chromosome components play in radiation damage could be answered. In order to carry out this research, a system has to be found that can provide an ample number of chromosomes, cytological or genetic markers, and in which it would be possible to identify and measure radiation products. In particular, it would be desirable to see if there is a correlation between the induction of thymine dimers in DNA[1] and in vivo induction of chromosomal aberrations with ultraviolet light.[2]

Results. – 5-Aminouracil (5-AU) was used to partially synchronize *Vicia faba* root tip meristematic cells and obtain a high proportion of the cells in mitosis. Cell-free preparations of metaphase chromosomes and interphase nuclei were obtained by fixing roots with 95% alcohol–acetic acid (3:1) or 2% neutral formalin,[3] and then homogenizing the tissue with Dry Ice powder. Excellent

morphological chromosome structure is maintained by these methods. However, chromosomes isolated after formalin fixation are held together in clumps. Attempts to separate pure preparations of chromosomes from nuclei and cytoplasm by density gradient centrifugation in sucrose or 2-bromoethanol have not yet succeeded.

Preliminary enzymatic digestion studies on the crude samples of fixed metaphase chromosomes are summarized in Table 1.16.1. The actions of deoxyribonuclease and ribonuclease were checked with Feulgen and azure-B bromide stains.

Table 1.16.1. Appearance of Fixed Metaphase Chromosomes After Enzymatic Treatment

Enzyme	3:1 Fixed	2% Formalin Fixed
Deoxyribonuclease	Swollen	Swollen
Ribonuclease	No effect	No effect
Trypsin	Dissolved	Stretched but not broken

Crude preparations of thymidine-labeled *Vicia* chromosomes were irradiated with ultraviolet light (2800 A, 4200 ergs/mm^2). Two percent of the labeled thymidine was detected in the form of dimers.

Discussion. – Results of enzymatic digestion on fixed *Vicia* chromosomes show that deoxyribonuclease and ribonuclease do not destroy the longitudinal integrity of the chromosomes. The protein matrix is sufficient to keep chromosomes intact. Chromosomes, fixed in 2% formalin, were elongated after treatment but no breaks were visible. Whether all the histones, which are not lost during fixation, are removed by the trypsin is not known. Possibly, the nucleoprotein complex and trypsin specificity hindered the ability of trypsin to dissolve the chromosome. Chromosomes, isolated in 3:1 fixative which removes histones, were dissolved with trypsin.

The finding of thymidine dimers in ultraviolet-irradiated isolated chromosomes indicates that the energy absorbed by the complex metaphase chromosome can be utilized to form thymine dimers. The formation of thymine dimers, in itself, does not lead to breaks in fixed metaphase chromosomes. This might be interpreted as indicating a time lapse and some metabolism are needed between absorption of ultraviolet light and chromosome breakage.

[1] R. B. Setlow, P. A. Swenson, and W. L. Carrier, *Science* **142**, 1464–65 (1963).

[2] E. H. Y. Chu, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 47–48.

[3] J. Mcleish, *Proc. Roy. Soc. (London) Ser. B* **158**(971), 261–78 (1963).

1.17 FURTHER EVIDENCE FOR MUTAGEN SPECIFICITY IN *NEUROSPORA*[1]

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

Introduction. – Do different mutagenic agents produce a different spectrum of induced mutation? In a previous report[2] data were presented showing a striking correlation between the properties of mutations induced in the *ad-3* region of *Neurospora* and mutagenic origin. A direct correlation was found in the percentages of leaky *ad-3A* and leaky *ad-3B* mutants in samples of mutants occurring spontaneously or those obtained after treatment of wild type with x rays, ultraviolet (UV), nitrous acid (HNO_2), 2-aminopurine (2AP), ethyl methanesulfonate (EMS), or mesodiepoxybutane (DEB). Marked differences were also found in the percentages of *ad-3B* mutants showing allelic complementation in each of the samples. Furthermore among the *ad-3B* mutants a direct correlation was found between the differences in the percentages of leaky mutants and the percentage of mutants showing allelic complementation.

An analysis of the complementation patterns of individual *ad-3B* mutants has recently been completed. It not only provides further evidence for mutagen specificity but various correlations between the new data and those reported previously[2] suggest that it may be possible to distinguish base-pair substitution from base-pair addition or deletion mutations by the contrast in their phenotypic effects.

Results. – Complementation patterns of individual *ad-3B* mutants were determined with a set of 17 tester strains derived from a series of HNO_2-induced mutants that form a linear complementation map of the *ad-3B* cistron consisting of 17 complons (or complementation subgroups). Complementation tests were made on a total of 564 mutants under identical conditions described previously.[3] Of the 107 different complementation patterns detectable with the present tester set, 52 different patterns were detected among the 530 *ad-3B* mutants that could be mapped. In addition, marked differences were found not only in the spectra of complementation patterns but also in the average number of complons covered by those mutants showing allelic complementation. The percentage of complementing mutants covering from 1 to 16 complons in each sample is shown in Fig. 1.17.1. Each sample of mutants shows a different type of distribution of complementation patterns, and the average complon coverage for all of the complementing mutants in each sample varies as follows: spontaneous, 12.6; x ray, 11.9; UV, 7.8; 2AP, 4.4; HNO_2, 6.4; EMS, 7.2; and DEB, 9.4. The number of complons covered by all of the mutants in this mixed sample shows a pronounced bimodal distribution. The average number of complons covered by those mutants with *restricted-type* complementation patterns (covering one to ten complons) is 4.3, and the average number of complons covered by those mutants with *extended-type* complementation patterns (covering 11 to 16 complons) is 13.4. Of the mutants in the group with unknown complementation patterns, 33/34 are leaky mutants that grow so rapidly alone that it is difficult to determine their complementation patterns with any certainty. The remaining mutant is positive with all testers and probably is the sole representative of a new complementation subgroup in the *ad-3B* cistron.

Discussion. – Mutagens that alter DNA by producing base-pair substitutions can have two different effects. If the code is altered so that the resulting triplet makes sense, then a different

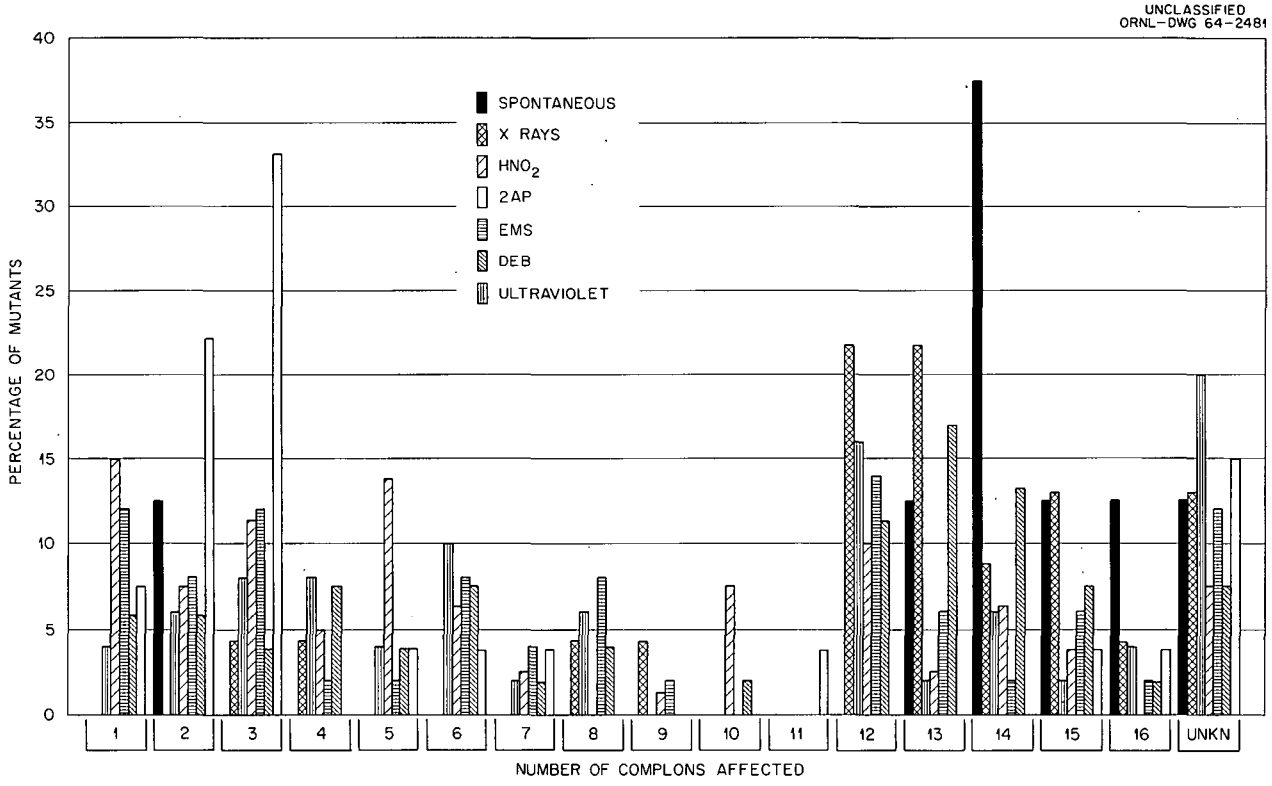

Fig. 1.17.1. Percentage of Mutants Covering from 1 to 16 Complons on the Complementation Map of the *ad-3B* Cistron in Samples of Diverse Mutagenic Origin.

amino acid is specified for that position in the polypeptide chain; if the altered triplet does not make sense, then no amino acid is specified. The ratio of these two effects would depend on the degeneracy of the code. Mutants produced by agents that produce only base-pair substitutions could specify, predominantly, polypeptide chains with only a single amino acid substitution. The effect of erroneous single amino acid substitutions at various positions along the chain should have a marked effect on the specific activity of the enzymes. Many enzymes should have only a slight change in surface charge and/or tertiary structure that would lead to a concomitant reduction in specific activity. In many cases this might lead to incomplete rather than complete blocks in the biosynthetic pathway. In addition to a high incidence of leaky mutants, a large proportion of base-pair substitution mutants should show allelic complementation, and, under ideal conditions, individual mutants should cover only one or a few complons on the complementation map. The opposite should be true of mutations resulting from single base-pair addition or deletion. Such alterations should produce "reading frame mutants" in *Neurospora*, as in phage,[4] and these mutants are thought to specify either a drastically altered polypeptide chain or none at all. One would expect that such mutants would not be leaky, and if they complement at all would react with only a limited number of tester strains. Furthermore, individual mutants would cover many complementation subgroups on the complementation map.

The percentages of mutants with extended- or restricted-type complementation patterns were found to vary with mutagenic origin as shown in Table 1.17.1. It is of particular interest that the

Table 1.17.1. Percentages of Mutants with Extended-Type (11 to 16 Complons) or Restricted-Type (1 to 10 Complons) Complementation Patterns Among *ad-3B* Mutants of Different Mutagenic Origin Showing Allelic Complementation

Mutagenic Origin	Complementation Pattern			
	Extended-Type		Restricted-Type	
	Number	Percentage	Number	Percentage
Spontaneous	6/7	85.7	1/7	14.3
X ray	16/20	80.0	4/20	20.0
UV	15/40	37.5	25/40	62.5
2AP	3/23	13.0	20/23	87.0
HNO_2	18/74	24.3	56/74	75.7
EMS	15/44	34.1	29/44	65.9
DEB	27/49	55.1	22/49	44.9

Table 1.17.2. Percentages of Leaky *ad-3B* Among Noncomplementing Mutants or Those with Restricted- or Extended-Type Complementation Patterns

Mutagenic Origin	Complementation Pattern			
	Extended-Type (11 to 16 Complons)	Restricted-Type (1 to 10 Complons)	Noncomplementing (17 Complons)	Unknown
Spontaneous	0/6	0/1	0/13	1/1
X ray	0/16	0/4	0/69	3/3
UV	0/15	6/25	0/61	10/10
2AP	0/2	9/21	0/8	4/4
HNO_2	0/18	20/56	0/31	6/6
EMS	0/15	1/29	0/44	6/6
DEB	0/27	7/22	0/47	3/4
Totals	0/99	43/158	0/273	33/34

highest percentages of mutants with restricted-type complementation patterns are found among the samples induced by 2AP and HNO_2. These mutagens are known to produce predominantly base-pair substitutions in the form of AT to GC and GC to AT transitions in phage. Similarly the highest percentage of mutants with extended-type complementation patterns is found in the sample of spontaneous and x-ray-induced mutants. Both base-pair deletion and addition are known to occur spontaneously in T_4 bacteriophage.[4] It is well known that x rays cause interstitial deletions as single-hit events, but this effect has not as yet been analyzed on a molecular level. Another correlation of interest is one between the type of complementation pattern and the incidence of leaky mutants as shown in Table 1.17.2. All of the 43 leaky mutants have restricted-type complementation patterns; none are found among the mutants with extended-type patterns or in the noncomplementing group.

The present and previous data[2] on the properties of *ad-3* mutants produced by different mutagens indicate a direct correlation between the phenotype and the type of genetic alteration. It seems logical to conclude that many of the leaky mutants with restricted-type complementation patterns result from base-pair substitutions, whereas those nonleaky noncomplementing mutants or nonleaky mutants with extended-type complementation patterns result from base-pair addition or deletion. Studies on the specific reversibility of HNO_2-induced mutants was discussed in a previous report,[5] and evidence was presented indicating that these mutants include those arising from GC to AT and AT to GC transitions. A similar analysis of noncomplementing *ad-3B* mutants, in addition to those with restricted- and extended-type complementation patterns, is in progress to further test these conclusions.

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1.18 INHIBITION OF DNA SYNTHESIS AND MITOSIS IN MAMMALIAN CELLS BY ULTRAVIOLET AND X RAYS

E. H. Y. Chu
Patricia G. Lankford
Barbara A. Barbee
Charlotte M. Boone

Introduction. – In spite of considerable research into the radiation-induced mitotic inhibition in cells of various organisms, relatively few investigations have given definitive information on metabolic changes when cell division is temporarily or permanently impaired. We have compared the cellular effects produced by ultraviolet and x rays in cultivated mammalian cells. Our results permit certain conclusions to be drawn concerning the extent of injury and the time and pattern of recovery with respect to wavelength and dose. These results will be correlated with our studies, now in progress, on stage sensitivity of radiation-induced chromosome aberrations in the same cell system.

Material and Methods. – Clonal cell cultures derived from Chinese hamster embryonic tissues were irradiated with monochromatic ultraviolet light or 250-kv x rays, with or without prior H^3-thymidine pulse (15 min) labeling. Mitotic index and percentage of labeled cells (metaphase and interphase) were determined at various times after irradiation.

Results and Conclusions. – With either x rays or ultraviolet, cell division in progress was not stopped immediately after irradiation, although stickiness and swelling of metaphase chromosomes and other cellular abnormalities were observed. With 150 r of x rays, very few mitotic figures were observed within the first 2 hr after irradiation, but the total reproductive capacity did not appear to be reduced. The peak of mitotic activity, however, was reached later in the treated than in the untreated material. On the other hand, an incident exposure of ultraviolet (100 ergs/mm^2 at 2652 A) not only delayed the appearance of mitotic figures for at least 6 hr but also permanently sterilized the majority of cells. Chromosome aberration studies revealed that, at this dose, a few cells did not divide until as much as 96 hr later. The severeness of inhibition increases rapidly with dose.

DNA synthesis was postponed for approximately 2 hr when cells were irradiated with either ultraviolet or x rays at the above doses. Unlike the x-irradiated cells, the proportion of ultraviolet-irradiated cells capable of resuming DNA synthesis was considerably reduced.

Drastic inhibition of mitosis can be induced by ultraviolet at wavelengths over the broad range of 2400 to 2800 A, indicating that inhibition of mitosis is caused by a mixture of many specific types of damage to the cell constituents and that it is not caused by the incapacitation of DNA synthesis alone. Ultraviolet-induced mitotic delay is also found to be non-photoreactivable.

Depending on the wavelength, three general patterns of recovery to mitotic activity were observed in ultraviolet-irradiated cells: (1) initial inhibition followed by division synchrony (2250 A, 2378 A); (2) prolonged delay or permanent sterilization (2652 A, 2804 A); and (3) possible stimulation (3130 A, 3650 A).

1.19 RANDOMNESS OF INDUCED CHROMOSOME BREAKS BY ULTRAVIOLET AND 5-BROMODEOXYURIDINE

Tien-Ding Chang E. H. Y. Chu

Introduction. – Hsu and Somers[1,2] reported that replacement of thymidine moieties of DNA by 5-bromodeoxyuridine (BUdR) induces breakage in several specific (heterochromatic) regions of mammalian chromosomes. They postulated that these vulnerable regions contain relatively high ratios of adenine-thymine (A-T) base pairs. We have performed similar experiments in order to test this attractive hypothesis which correlates a known chemical action with morphological manifestation at another level of magnification. On the basis of our earlier finding[3,4] that 5-BUdR pretreatment greatly increases the frequency of ultraviolet-induced chromosome aberrations, we additionally wanted to know if combined treatments also increased the breakage rate at the same specific regions. The distribution of ultraviolet-induced breaks along chromosomes was also tested.

Material and Methods. – A clonal, quasi-diploid cell line originated from Chinese hamster cells was treated for 48 hr with 10^{-5} *M* BUdR before ultraviolet irradiation (100 ergs/mm^2 at 2652 A). Metaphase figures were accumulated by colchicine from 6 to 24 hr after irradiation. Three morphologically distinct chromosomes (a normal No. 1, a slightly modified No. 1, and an unusually long acrocentric) were chosen for scoring aberrations at designated chromosome regions. Stringent criteria were adopted in defining enhanced secondary constrictions (isolocus constriction on both chromatids), achromatic lesions (unstained or faintly stained gaps, sometimes with connecting "thread"), and true chromatid breaks (terminal deletions with displaced fragments, isochromatid breaks, and chromatid exchanges).

Results and Discussion. – Table 1.19.1 summarizes the frequency distributions of chromatid aberrations in specific regions of chromosome No. 1. This submetacentric chromosome is the longest in the complement and normally has a secondary constriction in the proximal region (4) of its longer arm. In 5-BUdR-treated cells, a significantly higher number of cells show a lengthening of the secondary constriction and achromatic lesions at the same site. This agrees with the observations of Hsu and Somers.[1,2] Unless we also combine all kinds of abnormalities observed in this region (4), as these authors apparently have done, we have no evidence for the induction by 5-BUdR of localized true chromatid breaks.

Ultraviolet induces both achromatic lesions and chromatic aberrations, but few, if any, stretched primary or secondary constrictions. The distribution of breaks appears to be random. The present study confirms and extends our earlier finding that incorporation of 5-BUdR increases ultraviolet radiosensitivity in terms of chromosome breaks, but it does not provide evidence for the increase of radiosensitivity at specific chromosome regions.

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[2] C. E. Somers and T. C. Hsu, *Proc. Natl. Acad. Sci. U.S.* **48**, 937 (1962).

[3] E. H. Y. Chu, *Proc. Intern. Congr. Genet., 11th, The Hague, Netherlands, 1963*, vol. 1, p. 85.

[4] E. H. Y. Chu, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 47–48.

Table 1.19.1. Frequencies of Enhanced Secondary Constriction, Achromatic Lesions, and Chromatid Breaks at Ten Equally Spaced Regions of Chinese Hamster Chromosome No. 1 Following 5-BUdR and Ultraviolet Treatment

Treatment[a]		Chromosome Region										Total Cells Examined
		1	2	3	4	5	6(*K*)[b]	7	8	9	10	
Untreated	A	0	0	3	0	0	0	0	1	1	0	1000
	B	0	0	0	0	0	0	0	0	0	0	
5-BUdR	A	0	4	2	63	0	0	1	1	0	0	1734
	B	0	2	1	5	0	1	0	1	0	0	
UV	A	1	20	20	17	3	1	8	3	4	0	1871
	B	0	6	11	5	3	1	3	1	0	0	
5-BUdR + uv	A	1	6	6	18	2	1	2	1	8	0	610
	B	0	4	3	4	2	1	3	2	1	0	

[a]A, enhanced secondary constriction and achromatic lesions. B, true chromatic breaks.

[b]*K*, kinetochore region.

1.20 COMPETITIVE GROWTH OF HEMATOPOIETIC CELLS IN LETHALLY IRRADIATED MICE

R. A. Popp Diana M. Popp

Introduction. – When mixtures of bone marrow cells from each of two strains of mice are injected into a lethally irradiated mouse, the recipient's hematopoietic system is repopulated by one of the two types of donor marrow.[1] Which of the two types of bone marrow continues to function depends upon the strains of origin of the donor marrows and the strain of the recipient. The relative efficiencies of bone marrow and of fetal liver from a few strains of mice to grow in F_1 and homologous recipients have been examined.

Methods and Results. – Adult bone marrows or fetal livers from 16-day fetuses were mixed prior to injection into lethally irradiated recipients. Twenty million cells were transferred to each recipient. Strains of mice and the donor-recipient combinations used are given in Table 1.20.1. The strain of origin of the hematopoietic cells that repopulated the recipient was determined from analysis of the hemoglobin type and/or the red cell serotype (lymph node serotype in a few cases) at 30, 60, and 90 days or later after the injection.

Discussion. – Mixtures other than ten million cells from each donor were used for each donor-recipient combination to determine the degree to which the ratio could be changed before the less efficient marrow repopulated the recipient. The results suggest a kind of hierarchy. Of primary importance, hematopoietic cells from strain 101 and C_3H (H-2^k) mice are more efficient than those from BALB/c, B10.D2, and DBA/2 (H-2^d) mice, which are more efficient than marrow cells from A.BY, C57BL/6, and C57BL (H-2^b) mice. Secondly, when marrow cells from mice of the same H-2 type are used, erythrocytes from both types of marrow may be found in the 60- to 90-day chimeras; however, proliferation of one of the donor cell types ceases within 180 days after marrow transfer. Fetal liver hematopoietic cells also showed marked

Table 1.20.1. Relative Abilities of Mixtures of Hematopoietic Cells to Grow in Lethally Irradiated Recipients

Strains of Origin of Donor Cells		Recipient	Erythrocyte Type Found in 60- to 90-Day Chimeras
		Bone Marrow	
C57BL	101	$B1F_1$	101
C57BL	101	BC_3F_1	101
C57BL	101	CBF_1	101
C57BL	BALB/c	CBF_1	BALB/c
C57BL	A·BY	$1C_3F_1$	A·BY
C57BL	DBA/2	CBF_1	DBA/2
DBA/2	C57BL/6	$B_6D_2F_1$	DBA/2 or C57BL/6[a]
DBA/2	B10·D2	$B_6D_2F_1$	DBA/2 and B10·D2[b]
BALB/c	C57BL/6	CBF_1	BALB/c
B10·D2	C57BL/6	$1C_3F_1$	B10·D2
B10·D2	C57BL	$1C_3F_1$	B10·D2
A·BY	DBA/2	$1C_3F_1$	DBA/2
A·BY	DBA/2	$B_6D_2F_1$	DBA/2
A·BY	101/S[c]	$B1F_1$	101/S
101/S	DBA/2	D_21F_1	101/S
101/S	C_3H	$1C_3F_1$	101/S and C_3H[b]
		16-Day Fetal Liver	
BALB/c	C57BL	CBF_1	BALB/c
DBA/2	C57BL/6	$B_6D_2F_1$	DBA/2 and C57BL/6[b]

[a]DBA/2 outgrows the latter, but C57BL/6 chimeras were frequent.

[b]In 60-day chimeras, both cell types were found; however, cells of the former strain gradually outgrow those of the latter.

[c]Strain 101/S is coisogenic with 101; the former has a single and the latter a diffuse hemoglobin.

differences in their abilities to repopulate lethally irradiated F_1 recipients.

[1]R. A. Popp *et al.*, "Relative Ability of Parental Marrows to Repopulate Lethally Irradiated F_1 Hybrids," to be published in *Ann. N.Y. Acad. Sci.*

1.21 ISOLATION OF SERUM ESTERASE IN MICE

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R. E. Canning[1]

Introduction. – A single band of esterase in C57BL and C57L mice and two esterases in BALB/c, 101, and SEC mice, all electrophoretically similar to serum albumin, have been shown to be codominantly inherited in F_1 progeny and to be controlled by alleles at a locus, *Es*.[2] Studies on the substrate specificities and gene-controlled molecular alterations await efficient methods for the isolation of relatively large amounts of the enzyme.

Methods and Results. – 101 male serum was fractionated on a DEAE-cellulose column by gradient elution with sodium phosphate and sodium chloride. The eluents used were: (1) 0.005 *M* sodium phosphate, pH 7.0; (2) 0.02 *M* sodium phosphate, pH 6.0; (3) 0.05 *M* monosodium phosphate, pH 4.5; (4) 0.05 *M* monosodium phosphate and 0.02 *M* sodium chloride, pH 4.5; (5) 0.05 *M* monosodium phosphate and 0.05 *M* sodium chloride, pH 4.5; (6) 0.05 *M* monosodium phosphate and 0.10 *M* sodium chloride, pH 4.5. Each fraction was analyzed for protein concentration and esterase activity.[3] After dialysis against distilled water and lyophilization, the protein and esterase fractions were identified by starch gel electrophoresis.[4] The separation of esterase from albumin appeared incomplete (Fig. 1.21.1) and ultracentrifuge data showed that some macroglobulin remained also. However, by equilibration of the DEAE-cellulose and application of the serum with the first buffer, by deletion of the second buffer, and by quadruplication of the fourth buffer, a better separation of esterase from albumin was obtained (Fig. 1.21.2). At this point the source of serum was changed from strain 101 mice, to C57BL with a single esterase, to facilitate isolation. The use of stepwise elution with acetic acid–sodium acetate buffer on CM-cellulose removed some of the macroglobulin appearing in the DEAE-cellulose fraction, but the recovery of the enzyme was greatly reduced. Sephadex G-200, using 0.04 *M* sodium phosphate buffer, pH 6.8, effectively separated the macroglobulin from the esterase fraction. Thus, a relatively pure sample of esterase can be obtained. Ultracentrifuge data indicate that the esterase has a molecular weight of approximately 70,000, which is similar

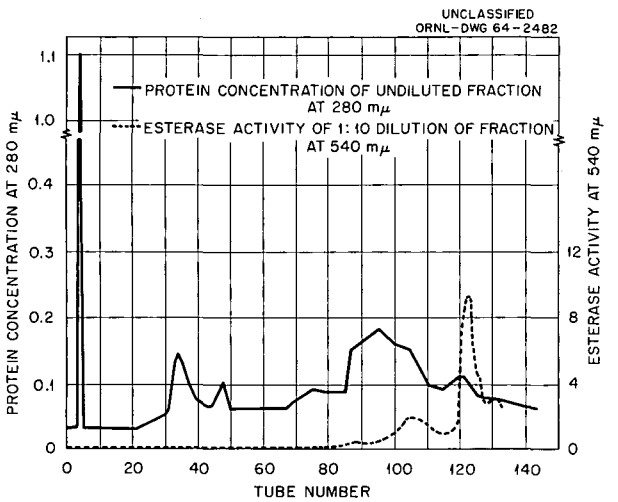

Fig. 1.21.1. Protein and Esterase Separation of 3.0 ml of 101 Male Serum on DEAE-Cellulose. Each fraction tube contained 12 ml of eluate.

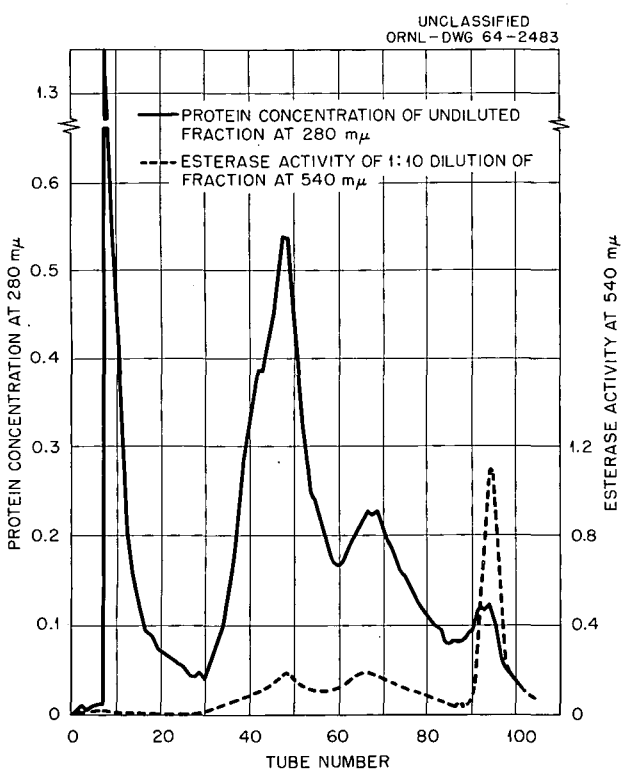

Fig. 1.21.2. Protein and Esterase Separation of 5.5 ml of C57BL Male Serum on DEAE-Cellulose. Each fraction tube contained 12 ml of eluate.

to albumin. Guanidine hydrochloride treatment did not dissociate the enzyme into smaller components, indicating that it is not an isozyme.

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1.22 AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF MOUSE HEMOGLOBINS

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Introduction. – Studies on the primary structure of mouse hemoglobins are being continued using methods previously described.[1]

Results. – The amino acid composition of the two insoluble (core) peptides of the α chain of C57BL hemoglobin is Lys_2 His_4 Asp_3 Thr_4 Ser_6 Pro_2 Ala_5 Val_4 Leu_7 Phe_2 Cys. Thus, the α chain is composed of 141 amino acids. The sequence of the amino acids in each of the 14 α-chain peptides is presently being determined by Edman degradation procedures.[2] The tryptic peptides of the α chain of a third hemoglobin (NB) have been analyzed for their amino acid content. Compared with C57BL, αT-4 of NB has a Val substitution for one of the Gly residues and αT-9 has one less Asp and Val residues that are replaced by Ser and Ileu. The exact positions of these amino acid substitutions remain to be established.

Discussion. – Amino acid substitutions were expected to be correlated with the solubility differences among C57BL ($Hb^1/Hb^1;Sol^1/Sol^1$), HBS ($Hb^1/Hb^1;Sol^2/Sol^2$), and NB ($Hb^1/Hb^1;Sol^3/Sol^3$) hemoglobins. Amino acid differences were found in the α chains of the above hemoglobins. The substitutions in αT-4 and αT-9 of NB compared to C57BL hemoglobin make it possible to detect crossing over within the α-chain controlling locus.

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2. Drosophila Cytology and Genetics

D. L. Lindsley

Radiation Genetics (2.1–2.6)

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Ruby M. Valencia[a,c]
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Claire J. Witten
B. J. Williams[d]
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Chromosomal Behavior and Biochemical Genetics (2.7–2.10)

E. H. Grell
Rhoda F. Grell
P. A. Roberts
D. G. Davis[e]
Ruby D. Wilkerson

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[b]University of Buenos Aires School of Sciences, Argentina.
[c]National Science Foundation Grant; on leave from the Argentine Atomic Energy Commission.
[d]Loanee.
[e]Research associate.
[f]Consultant.

Pseudoallelism and Immunogenetics (2.11)

W. J. Welshons
Bobbie J. Scandlyn
Elizabeth S. Von Halle[f]

2.1 TOTAL RADIATION DAMAGE TO ENTIRE GENOMES

Ruby M. Valencia
J. I. Valencia
D. L. Lindsley
E. R. Muñoz

Numerous studies have been made of the effects of radiation upon genes and chromosomes, but the total damage done to the whole genome has never been adequately determined. In spite of the obvious technical difficulties in carrying out such an experiment, an attempt is now being made to assess genetic damage in entire X-bearing and Y-bearing genomes irradiated in mature spermatozoa of *Drosophila melanogaster*. X rays (250 kvp, 30 ma with 3-mm aluminum filter) have been used and most of the work has been done with 4000 r delivered in $2\frac{1}{2}$ to 3 min. A few genomes were given 2000 r. The genetic effects being screened for are lethals and sterility factors, morphological mutations, and alterations in chromosome order which are detectable by genetic tests and/or microscopic examination of salivary gland chromosomes (translocations, inversions, deficiencies, etc.).

A considerable part of the effort thus far expended has gone into preparing the special stock from which the males to be irradiated are taken and those stocks to be used in balancing and analyzing the irradiated genomes. The irradiated males have the following genetic constitution: *y cho*/B^SY; *red sbd*; *ci*. The males are irradiated and mated to females bearing genetic markers and/or crossover suppressors in all chromosomes and also carrying a Y chromosome marked with y^+, as follows: FM6, y^{31d} sc^8 *dm B*/In(1)AM, y^2/y^+sY; *pr Bl*/SM1, al^2 *Cy cn* sp^2; Tp(3R)Vno, *Vno*/In(3LR)Ubx^{130}, *Ubx*^{130} e^s; *pol*. The mutants *cho* and *red*, as well as *cn*, present on the balancer chromosome II, are larval markers to facilitate choosing the desired larvae for salivary analyses. The mutants *y* and *ci* are markers used in the translocation test, for which females carrying the irradiated genome in heterozygous condition with

the balancing chromosomes are crossed to *f*/Y; *bx e*; pol males.

For X-bearing genomes, daughters of the irradiated males are collected which received the FM6, Y, *Cy*, *Ubx*, and *pol* chromosomes from the mother and the irradiated X and autosomes from the father. For Y-bearing genomes, nondisjunctional daughters are collected which received the two X chromosomes from the mother and the irradiated B^SY from the father. Their autosomal constitution is the same as that of the X-bearing genomes. Both kinds of female are collected as virgins and mated individually to males whose autosomes are like those of the P1 female but whose X and Y chromosomes are normal. This procedure was necessary in order to use balancer males of adequate viability and fertility.

Most of the irradiated genomes which did not cause sterility or semisterility of the original F1 daughter have been saved in their entirety, usually in one stock, although not necessarily so. In some cases the different chromosomes of the genome have been kept in two (rarely three or four) stocks. In a few cases individual chromosomes of the genome were lost, and this usually appeared to be due to the inviability of the chromosome, although in a very few cases it may have been accidental. Table 2.1.1 shows the number of whole and partial genomes now being studied. The numbers of chromosomes in stock and the information to date as to their viability and fertility are shown in Table 2.1.2. Translocation tests and cytological analyses have only recently gotten under way, so that information on chromosome rearrangements is not sufficient to warrant presentation at this time. Some translocations (approximately 60) are suspected on the basis of observations made during the course of stock

Table 2.1.1. Number of Genomes Being Studied

Type of Genome and Dose	Number of F1 Females Mated	F1 Females Sterile or Semisterile	Females Fertile but Genomes Not Saved	Whole Genomes Saved	Partial Genomes Saved
X-bearing, 2000 r	42	0	0	41	1
X-bearing, 4000 r	240	24	8	190	18
Y-bearing, 4000 r	163	16	5	132	10

Table 2.1.2. Chromosome Viabilities

Dose (r)	Chromosome	Number in Stock	Number Lethal	Number Semilethal	Viable				Viability Not Determined	Fertility Not Determined
					♂ Sterile	♀ Sterile	♂ and ♀ Fertile	♂ and ♀ Presumed Fertile		
2000	X	42	2	0	0	0	40	0	0	
	II	42	7	4	0	0	0	29	2	
	III	41	9	6	0	0	0	23	3	
	IV	42	0	0	0	0	0	39	3	
4000	X	208	16	3	9	1	98	77	4	
	Y	143			18		114			11
	II	339	75	6	0	0	0	174	84	
	III	333	70	14	0	0	0	135	114	
	IV	341	3	2	0	0	0	172	164	

making. At present only four of these have been observed cytologically and all four were confirmed. One unsuspected X-IV has been found through cytological observation.

It is considered that the results obtained to date demonstrate that it will be possible by this method to obtain useful information on radiation-induced genetic damage to entire genomes.

2.2 A SEARCH FOR SEX-LINKED RECESSIVE LETHALS IN *DROSOPHILA MELANOGASTER* THAT CAN BE TRANSMITTED BY TRIPLOID FEMALES BUT NOT BY DIPLOID DAUGHTERS

H. C. Armentrout

Introduction. – An attempt was made to find an explanation for the high degree of sterility of variants induced by irradiation of stage 14 oocytes. This observation was made earlier during an experiment concerned with determining the effects of x rays in producing visible mutations at specific loci.[1] It was postulated that perhaps the events that lead to recessive visible mutation in irradiated stage 14 oocytes might at the same time lead to dominant female sterilizing changes. If this is the case, then one might assume that (1) recessive lethal mutation and dominant sterile mutation changes might also be caused by the same event, (2) this correlation might also exist for mutations induced in mature spermatozoa, and (3) in a fraction of the cases the sterilizing effect might be dominant to one normal genome but recessive to two in triploid females. Accordingly, an attempt was made to detect sex-linked recessive lethals that can be transmitted by triploid but not by diploid females.

Methods and Materials. – Males, which carried a multiple inverted X chromosome (FM6) to prevent crossing-over and marked with yellow (*y*), diminutive (*dm*), and Bar (*B*), were x-irradiated and mated to virgin triploid females. The triploid females had a compound X chromosome which was homozygous for *y*, scute (*sc*), apricot (w^a), and echinus (*ec*), and FM6 as the free X chromosome. Virgin F_1 triploid females, which possessed the irradiated free X chromosome and the compound X chromosome, were crossed individually to Oregon-R males. The progeny were screened for *y dm B* males. If no such males were found, the diploid females bearing the irradiated X chromosome were mated in mass to Oregon-R males. This cross was checked for fertility of the females and/or for confirmation of the lethality of the irradiated X chromosome.

Because the triploid females produced very few progeny, very often neither the male nor the diploid female having the irradiated X chromosome was present. Later in the experiment all classes from the crosses between the irradiated X chromosome-bearing triploid females and Oregon-R males were scored in an effort to determine if, in some cases, the irradiated X chromosome was lethal in diploids but not in triploids. These attempts were unsuccessful.

Two series of irradiations were made. The first series of experiments involved males which had received 2000 r, and the males of the second series received 4000 r. The males were taken out of the culture bottles after 48 hr.

Results. – The results of the two series of experiments are presented in Table 2.2.1.

Conclusions. – There was little correlation between sex-linked recessive lethals and sterility comparable to the observation that many of the variants produced in irradiated stage 14 oocytes

Table 2.2.1. Lethality and Sterility of the Progeny Produced by Triploid Females Bearing an Irradiated Free X Chromosome

Dose (r)	Number of Triploid Females Tested	Lethal Cultures		Lethal Cultures Producing Sterile FM6/OR Daughters	
		Number	Frequency	Number	Frequency
2000	1230	75	0.06	None	None
4000	939	116	0.12	3	0.02

were sterile. This means that the postulated dominant-sterile-recessive-visible type of induced change is not frequent, or that one or more of the assumptions that went into this particular test of the hypothesis were incorrect.

[1]R. M. Valencia, unpublished.

2.3 EFFECTS OF THE AMOUNT AND DISTRIBUTION OF X-CHROMOSOME HETEROCHROMATIN ON X-RAY-INDUCED X-AUTOSOMAL TRANSLOCATION FREQUENCIES

B. J. Williams

The metaphase length of the X chromosome can be varied from 0.63 to 1.37 times normal by manipulating the segment of proximal heterochromatin between the right breakpoints of In(1)sc^4 and In(1)sc^8. The same procedure also results in alteration of the distribution of the majority of X chromosome heterochromatin (see Fig. 2.3.1). Kaufmann[1] has shown that x-ray-induced breakability of the X chromosome is proportional to the metaphase length and yet Muller[2] has reported that In(1)sc^{4L},sc^{8R} and In(1)sc^{8L},sc^{4R} [= In(1)sc^{S1L},sc^{4R}] are equally involved in induced translocations. To investigate this paradox Muller's experiment has been repeated utilizing the four scute inversions shown in Figure 2.3.1. Males from these four scute stocks were irradiated with 4000 r and crossed to

$$\frac{\text{In(1)dl-49, B}^{M1}, sc\ v}{\text{In(1)dl-49, B}^{M1}, sc\ v}; \frac{Cy; Ubx\ e}{\text{T(2;3)bw}^{V4}}$$

females and, from this cross, flies of the genotype

$$\frac{\text{In(1)sc, } v\ B}{\text{In(1)dl-49, B}^{M1}, sc\ v}; \frac{Cy}{+}; \frac{Ubx\ e}{+}$$

were obtained and crossed to

$$\text{Ins(1)sc}^7, \text{AM/Y}; p^p\ sr\ bx\ e^s$$

males. Counts were made, and sequential analysis of the data was made using an IBM 7090 computer program described by Lindsley.[3]

The examination of 510 cultures disclosed 60 X-autosomal translocations. The numbers of lethals and (2;3) translocations were also recorded. These results are summarized in Table 2.3.1.

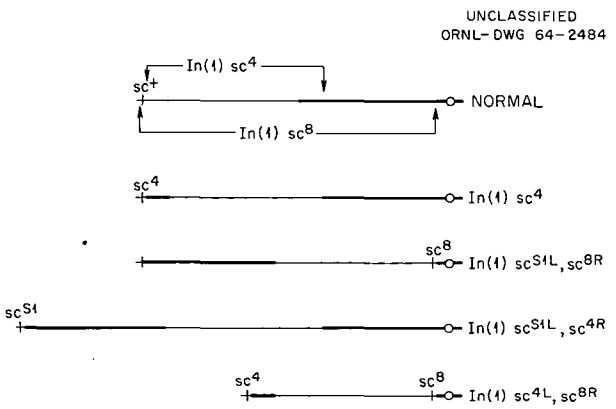

Fig. 2.3.1. Heterochromatic Constitution of the Various Scute Inversions Used in the Present Investigation. Heavy lines indicate heterochromatin; thin lines indicate chromatin.

Table 2.3.1. Incidence of Sex-Linked Recessive Lethal Mutations and Translocations Induced by 4000 r of X Rays in Different Scute Inversions

Irradiated Chromosome	Total Cultures Tested	Number of Lethals	Lethals (%)	T(2;3)	T(2;3) (%)	T(1;2)	T(1;3)	T(1;2;3)	T(X;A) (%)	T(X;A)/T(2;3)
In(1)sc^{S1L}sc^{8R}	191	26	13.6	16	8.4	7	9	5	10.2	1.2
In(1)sc^{S1L}sc^{4R}	134	22	16.4	9	6.8	5	8	1	10.4	1.5
In(1)sc^4	156	25	16.0	9	5.8	7	10	5	14.1	2.4
In(1)sc^{4L}sc^{8R}	29	6	20.7	3	10.3	1	1	1	10.3	1.0

The number of $In(1)sc^{4L},sc^{8R}$ cultures tested is very small and the results are not significant. The incidence of translocations observed for the other three chromosomes shows no correlation with heterochromatic content or distribution. Similarly the ratio of T(2;3) to T(X;A) and the incidence of sex-linked lethals show no relation to chromosomal constitution. The data seem to indicate that altering the distribution and amount of heterochromatin has no effect on the induction of X-autosomal translocations. These data are in agreement with those of Muller.[2] These data fail to support the supposition of Lindsley, Edington, and Von Halle[4] that the distribution rather than the amount of heterochromatin affects the incidence of induced rearrangements. They found that $In(1)sc^{8}$ and $In(1)sc^{S1L}$, sc^{4R}, which have a large segment of the proximal heterochromatin relocated distally, are more sensitive to x-irradiation, as measured by shift in sex ratio, than $In(1)sc^{4}$, which has only a small portion of the proximal heterochromatin terminally. The cytology of the translocations is being done and will probably also show that the amount and distribution of heterochromatin had no effects on the distribution of the breakpoints along the X chromosome. Some of the translocations have been tested for fertility, and of those tested roughly 65% are sterile, which is somewhat lower than the figure observed for X;autosome translocations involving X chromosomes in normal sequence.

[1]B. P. Kaufmann, *J. Exptl. Zool.* **102**, 293–320 (1946).

[2]H. J. Muller, *Genetics* **30**, 15 (1945).

[3]D. L. Lindsley, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp. 41–42.

[4]D. L. Lindsley, C. W. Edington, and Elizabeth S. Von Halle, pp. 63–76 in *Repair from Genetic Radiation (Leiden, 1960)* (ed. by F. Sobels), Pergamon, New York, 1963.

2.4 A REVISED EDITION OF *THE MUTANTS OF DROSOPHILA MELANOGASTER*

D. L. Lindsley E. H. Grell I. Lucille Norton

The reference book most used by *Drosophila* geneticists is *The Mutants of Drosophila melanogaster* by Bridges and Brehme. This volume contains descriptions of mutant phenotypes, the standard genetic locations of mutations, and cytological information. The collection of material for this book was completed in December of 1942. Since that date many new mutations and chromosomal rearrangements have been analyzed and a vast amount of information about old mutants has been collected. This material is scattered throughout the biological journals and much is unpublished. For several years there has been a need for a revised edition of the Bridges and Brehme publication. We have been gathering information for about a year for such a revision. All known *Drosophila* geneticists have been asked to submit pertinent information, and the literature has been searched. Procedures are being designed so that information can be processed automatically. At the present date we have about one-third of the volume in some stage of editing. We plan to be ready for publication in September of 1964.

2.5 MEIOTIC BEHAVIOR OF TANDEM COMPOUND RING CHROMOSOMES IN *DROSOPHILA*

L. Sandler[1] D. L. Lindsley

A tandem compound ring X chromosome (TRX) is equivalent to a tandem metacentric,[2] but with its free ends attached together to form a continuous ring.

The expectations from no, one, and two exchanges between the elements of such a compound have been presented in an earlier report.[3] At that time the results were preliminary and the models tested lead to inadmissible estimates of some of the parameters. The present data represent complete counts from TRX, *y cv v sd/y sn g* females carrying $y^{+} \cdot Y^{L}$ as a homolog. Equations for the TRX equivalent to those for TMX in the following paper[2] are as follows:

Regular patroclinous ♂♂ $= E_0 + E_1 + E_2$,

Matroclinous TRX ♀♀ $= E_0 + \frac{1}{2}(1 - c_1)E_1 + \frac{1}{16} E_2$,

Transposed TRX ♀♀ $= \frac{1}{16} E_2$,

Homozygous TRX ♀♀ $= \frac{1}{8} E_2$,

Single Ring ♀♀ $= \frac{1}{2} c_1 E_1 + \frac{1}{8}(1 + 2c_2)E_2$,

Equal Bridges $= \frac{1}{8} E_2$,

Lethals $= \frac{1}{2} E_1 + \frac{1}{4}(2 - c_2)E_2$,

where c_1 and c_2 represent the probability of recovering the single ring when a single ring separates from a double ring and a triple ring, respectively, at anaphase II, and E_i is the frequency of tetrads of rank i.

The results of the cross studied are presented in Table 2.5.1. According to the hypothesis that exceptional patroclinous males arise from equal double second anaphase bridges,[4] homozygous TRX females and exceptional patroclinous males should be recovered equally frequently. Actually there were 65 and 1127 respectively; thus there is a tremendous excess of exceptional patroclinous males. This cannot be attributed to a reduced recovery of TRX-bearing progeny, because any increase in this class leads to inadmissible estimates of parameters. Nor can it be attributed to primary nondisjunction of TRX from $y^+ \cdot Y^L$ as can be judged from the negligible frequency of TRX/$y^+ \cdot Y^L$ and $X^c/y^+ \cdot Y^L$ ova produced. Of 206 nonhomozygous TRX-bearing daughters that were test-crossed, 16 were transposed. Expectation based on the incidence of homozygosis was 12.2. Thus as in the case of TMX,[3] the frequencies of homozygotes and transposes are consistent with each other, but exceptional patroclinous males do not fit the hypothesis that they arise solely from equal double bridges at anaphase II. Assuming that this class arises from some fraction of the presumptive lethal classes, we may proceed with the solution of the equations. In order to do so it must be assumed that $c_2 = 1.0$; in the only other case in which a single X is known to separate from a triple X at A II the coefficient of nonrandomness can be shown to be 1.0.[5] With these provisions the estimates of the parameters are as follows: $E_0 = 0.154$, $E_1 = 0.672$, $E_2 = 0.174$, and $c_1 = 0.98$. These results accord well with those from TAX[5] and TMX.[2]

We have attempted to measure the recovery of TRX in the absence of exchange by the use of *c3G*, an autosomal recessive that virtually eliminates meiotic exchange. TRX/*Y; c3G/c3G* females produced 1062 patroclinous males and 350 TRX-bearing females while their TRX/*Y; c3G/+* sisters produced 2797 patroclinous males, 690 TRX-bearing females, and 892 X^c-bearing daughters. We have been unable to reconcile this low recovery of TRX in the absence of exchange with the model presented above or any other model that we can devise.

Table 2.5.1. Progeny Produced by the Cross

$$\frac{\text{TRX, } y\ cv\ v\ sd/sn\ g}{y^+ \cdot Y^L}\ ♀♀ \times \frac{Y^S X \cdot Y^L,\ In(1)EN,\ y\ B}{O}\ ♂♂$$

Genotype of Ovum	Number of Zygotes Scored
$y^+ \cdot Y^L$	7836
O	1127
Nonhomozygous TRX	1435
Nonhomozygous TRX/$y^+ \cdot Y^L$	5
Homozygous TRX	170
Homozygous TRX/O	0
Single ring	3096
Single ring/$y^+ \cdot Y^L$	4

[1]Department of Genetics, University of Washington.

[2]D. L. Lindsley and L. Sandler, see chap. 2.6.

[3]L. Sandler and D. L. Lindsley, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 51–54.

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

[5]L. Sandler and D. L. Lindsley, *Genetics* **48**, 1533 (1963).

2.6 MEIOTIC BEHAVIOR OF TANDEM METACENTRIC COMPOUND X CHROMOSOMES IN *DROSOPHILA*

D. L. Lindsley L. Sandler[1]

A tandem metacentric compound X chromosome (TMX) is composed of two euchromatically complete X chromosomes attached to a medially located centromere and arranged in tandem sequence so that the synaptic configuration is a spiral. A group of such compounds that were + *sn* + *g* +/*cv* + *v* + *sd* in constitution were synthesized for genetic analysis.

The various products of recombination between the elements of a TMX are diagramed in Fig. 2.6.1; the frequencies with which these products are recovered are summarized in Table 2.6.1. Because the TMX separates from its homolog (i.e., $y^+ \cdot Y^L$) at the first meiotic division, equal numbers of presumptive X-bearing and presumptive $y^+ \cdot Y^L$-bearing ova are produced; thus the information in Table 2.6.1 may be collected into the following equations.

Regular patroclinous ♂♂ $= E_0 + E_1 + E_2$,

Matroclinous TMX ♀♀ $= E_0 + \frac{1}{2}(1-c)E_1 + \frac{1}{16}E_2$,

Transposed TMX ♀♀ $= \frac{1}{16}E_2$,

Homozygous TMX ♀♀ $= \frac{1}{8}(3-2c)E_2$,

Ring ♀♀ $= \frac{1}{2}cE_1 + \frac{1}{8}(1+2c)E_2$,

Double Bridges $= \frac{1}{8}E_2$,

Single Bridges $= \frac{1}{2}E_1 + \frac{1}{4}E_2$,

where c is the coefficient of nonrandomness,[2] that is, the probability of recovering the single ring X chromosome in the egg nucleus when the ring separates from a TMX at anaphase II, and E_i is the frequency of tetrads of rank i.

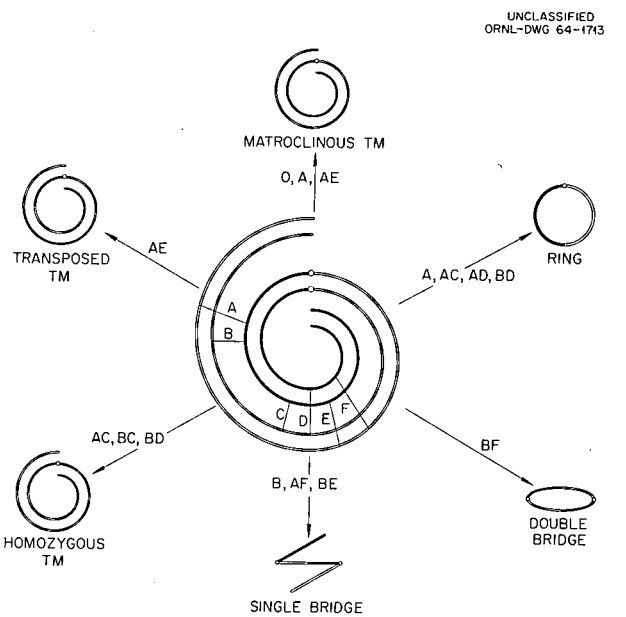

Fig. 2.6.1. Products of No, Single, and Double Exchange in Tandem Metacentric Compound X Chromosomes.

Table 2.6.1. The Consequences of Exchange in Tandem Metacentric Compound X Chromosomes

The crossovers involved are shown in Fig. 2.6.1

Rank and Frequency	Crossover Involved	Products of Anaphase II Separation and the Probability (in Parentheses) That They Will Be Included in the Functional Egg Nucleus				
E_0		Matroclinous TM	(1/2)	+	matroclinous TM	(1/2)
$1/2E_1$	A	Ring	(c)	+	matroclinous TM	(1 - c)
$1/2E_1$	B	Single bridge				
$1/8E_2$	AC	Ring	(c)	+	homogygous TM	(1 - c)
$1/8E_2$	AD	Ring	(1/2)	+	ring	(1/2)
$1/8E_2$	AE	Transposed TM	(1/2)	+	matroclinous TM	(1/2)
$1/8E_2$	AF	Single bridge				
$1/8E_2$	BC	Homozygous TM	(1/2)	+	homozygous TM	(1/2)
$1/8E_2$	BD	Ring	(c)	+	homozygous TM	(1 - c)
$1/8E_2$	BE	Single bridge				
$1/8E_2$	BF	Double bridge				

Novitski[3] has postulated that the double second anaphase bridges are excluded from the egg nucleus leading to the production of nullo-X ova which produce exceptional patroclinous males when fertilized by an X-bearing sperm. Thus from the equations it can be seen that there are three estimates of E_2: transposed TMX's, homozygous TMX's, and exceptional patroclinous males. In previous experiments either homozygosis[2] or exceptional patroclinous males[3] were scored, and the estimates of E_2 derived from these two scores were widely discordant. The current experiments were undertaken to obtain estimates of all three products of double exchange from the same cross in an attempt to resolve the discrepancies observed in previous experiments.

The results of a cross of TMX, *y cv v sd · sn g sc*[4] $y/y^+ \cdot Y$ ♀♀ × $Y^S X \cdot Y^L$, *In(1)EN, y B/O* ♂♂ are presented in Table 2.6.2. From the equations it may be seen that homozygous females should be (3 − 2c) times more frequent than exceptional patroclinous males (e.g., double bridges). The estimate of c derived from the data is 0.94; thus the ratio of homozygotes to exceptional patroclinous males should be 1.12, whereas it is observed to be 1.64. There are too few exceptional patroclinous males for the observed number of homozygous females. Transposed TMX's can be distinguished from matroclinous TMX's only by progeny testing. Of 153 females so tested 16 were transposed where 12.2 were expected on the basis of the incidence of homozygosis. Thus it is concluded that the incidences of homozygosis and transposition are mutually consistent but that exceptional patroclinous do not agree with expectation based on the hypothesis that they result from double second anaphase bridges. This conclusion is supported by similar data from other TMX chromosomes that we have investigated. Utilizing the homozygous TMX females, the nonhomozygous TMX females, the ring females, and the regular patroclinous males it is possible to estimate that $E_0 = 0.166$, $E_1 = 0.570$, $E_2 = 0.264$, and $c = 0.94$. These values agree reasonably well with those from the other tandem compound X chromosomes, for example, the tandem acrocentric[4] and the tandem ring.[5]

Table 2.6.2. Progeny Produced by the Cross

$$\frac{\text{TMX, } y\ cv\ v\ sd/y\ sn\ g}{y^+ \cdot Y^L}\ ♀♀ \times \frac{Y^S X \cdot Y^L,\ In(1)EN,\ y\ B}{O}\ ♂♂$$

Genotype of Ovum	Number of Zygotes Scored
$y^+ \cdot Y^L$	6230
O	143
Nonhomozygous TMX	1307
Nonhomozygous TMX/$y^+ \cdot Y^L$	7
Homozygous TMX	235
Homozygous TMX/$y^+ \cdot Y^L$	0
Ring	2267
Ring/$y^+ \cdot Y^L$	7

[1]Department of Genetics, University of Washington.

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

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

[4]L. Sandler and D. L. Lindsley, *Genetics* **48**, 1533 (1963).

[5]L. Sandler and D. L. Lindsley, see chap. 2.5.

2.7 REGULATION OF FOURTH CHROMOSOME NONDISJUNCTION BY THE LENGTH OF A FREE X DUPLICATION

Rhoda F. Grell

Introduction. – The fourth chromosome of *Drosophila melanogaster* occupies a special position in the genome, for, in addition to its extremely small size, it fails, under normal conditions, to undergo exchange. It has been shown that when the other *Drosophila* chromosomes are nonrecombinant, they become available for nonhomologous associations during distributive pairing.[1] Despite the absence of crossing-over, the fourth chromosome, apparently, does not become so involved. Failure to detect nonhomologous association of chromosome 4, unless one member of the pair is

effectively absent through involvement in a translocation, has led to the supposition that a special device, peculiar to the fourth chromosome, may be responsible for its regular behavior. The recent finding[2] that pairing preference during the distributive process is size dependent suggested the alternative explanation that it is the unique size of the 4 which safeguards it from heterologous involvement. The following experiment has been carried out in order to distinguish between the two possibilities.

Method and Materials. – If the failure of nonrecombinant heterologs to disturb the regular segregation of the 4's is due to the size disparity between the 4 and the rest of the genome, chromosomes more or less equal in length to the 4 should be effective in causing fourth chromosome nondisjunction. A series of free X duplication, whose mitotic metaphase length relative to chromosome 4 varies from $\leq$0.3 to 3.3, were introduced, one at a time, into females of the genotype y^2/y^2; ci^D/ey^D. The presence of y^+ on each duplication and of the dominant markers ci^D and ey^D on the two free 4's permitted the segregation of the three relevant chromosomes to be followed among the progeny. The ey^D phenotype is associated with a euchromatic duplication in chromosome 4 so that the ey^D chromosome is considered to be slightly larger than the nonduplicated ci^D chromosome. In a separate experiment, a translocation chromosome, T, with a mitotic length five to six times that of the 4, was substituted for a duplication. Nondisjunction values were calculated for the three chromosome sets: (1) ci^D/ey^D, (2) Dp, ci^D or T, ci^D, and (3) Dp, ey^D or T, ey^D. No viability corrections have been made, and since the doubly mutant class cy^D/ey^D is probably the least viable, nondisjunction values for the fourth chromosome are minimal ones.

Results and Discussion. – Nondisjunction frequencies for the three sets of chromosomes, plotted as a function of the length of the heterolog (Dp or T), are shown in Figure 2.7.1. The curve for fourth chromosome nondisjunction (ci^D, ey^D) shows that as the duplication approaches chromosome 4 in length, 4 nondisjunction steeply increases. At approximate equality with chromosome 4, 4 nondisjunction reaches a maximum of 36.3%, while nondisjunction between the duplication and ci^D is only 26.4%. At this point segregation of the heterologs Dp, ci^D is, therefore, more regular than that between the homologs ci^D, ey^D.

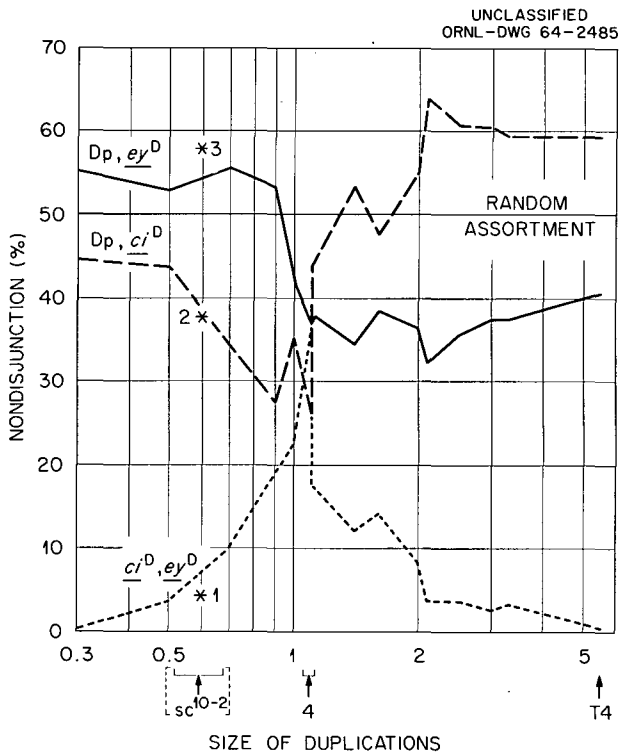

Fig. 2.7.1. Nondisjunction Frequencies for the Three Chromosome Sets (1) ci^D, ey^D; (2) Dp, ci^D or T4, ci^D; and (3) Dp, ey^D or T4, ey^D Plotted as a Function of Duplication (and T4) Length. *, nondisjunction frequencies obtained by Sturtevant with the sc^{10-2} duplication, where $*^1$ = ci^D, ey^D nondisjunction; $*^2$ = Dp, ci^D nondisjunction; $*^3$ = Dp, ey^D nondisjunction.

Beyond this point, as the duplications become larger than either 4, fourth chromosome nondisjunction abruptly decreases and tapers off with increasing duplication size.

As has been noted earlier, nondisjunction values in excess of 50% mean trivalents are occurring and it has been shown that disjunction from trivalents between three unequal heterologs follows a pattern in which the chromosome of intermediate length directs the small and large chromosomes to the same pole.[2] Here, we see evidence for the same phenomenon. With duplications smaller than 0.9 and greater than 2.0, trivalent formation is indicated by nondisjunction values greater than 50% (Fig. 2.7.1). If the ci^D fourth chromosome is somewhat smaller than the ey^D chromosome, for duplications smaller than the 4's, ci^D is the intermediate element and it is Dp, ey^D nondisjunction

which might be expected to exceed 50%; for duplications larger than the 4, ey^D is intermediate and *Dp*, ci^D nondisjunction might be expected to exceed 50%. In Fig. 2.7.1 we see that both expectations are met.

These results make it highly probable that the enigmatic segregation behavior of three fourth chromosomes described by Sturtevant[3] and known as "preferential segregation" arises from slight size differences among the fourth chromosomes and that it is these differences which determine their association and disjunction patterns at distributive pairing and lead to their nonrandom segregation. It appears, then, that the same behavior principles operate whether the three chromosomes involved are three heterologs, two homologs and a heterolog, or three homologs. It follows that homology is a negligible factor in distributive pairing.

Summary. – The fourth chromosomes are subject to the same rules of behavior during oogenesis as the other chromosomes of *Drosophila melanogaster*. In the diploid female the 4's are virtually always members of the distributive pool and will associate nonhomologously if heterologs of similar size are also present in the pool. When normal-size heterologs are all that are available, the small size of the 4's serves to insure regular fourth chromosome segregation.

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

[2]Rhoda F. Grell, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 54–56.

[3]A. H. Sturtevant, *Genetics* **21**, 444–66 (1936).

2.8 AN EXPLANATION FOR THE CORRELATED APPEARANCE OF DIVERSE KARYOTYPE ABNORMALITIES WITHIN HUMAN KINSHIPS

Rhoda F. Grell J. I. Valencia

The simultaneous occurrence of various kinds of karyotype abnormalities within a single human kinship such as (1) aneuploidy for an autosome associated with a translocation, (2) aneuploidy for the sex chromosome associated with autosomal translocation, and (3) aneuploidy for the sex chromosome associated with aneuploidy for an autosome has led a number of authors to suggest that these events are not fortuitous. Similar kinds of events have been observed in females of *Drosophila melanogaster* and the principles underlying them have recently been elucidated by the distributive pairing hypothesis.[1]

Application of these principles to human gametogenesis provides a satisfactory explanation for a number of puzzling cases described in the cytogenetic literature. A few typical cases are mentioned below.

Moorhead *et al.*[2] reported a case of a phenotypically normal woman with 45 chromosomes, who carried a translocation between chromosomes 13 and 22; her progeny included four severely retarded children, one triplo 21, and one normal. According to the interpretation proposed here, trisomy for chromosome 21 arose through the presence in the distributive pool of the minute translocated chromosome which must have been present in her germinal cells. Recently Hamerton[3] has reported a similar case, but the mother of the mongol was heterozygous for a translocation between two acrocentrics from the D group. Here too the small translocated chromosome could have paired with chromosome 21 to produce a gamete carrying two chromosomes 21 and lacking the translocation.

Several cases of the second category have been described by Lejeune.[4] In one, the mother and her XO daughter carried a 2-22 translocation. It is postulated that one of the translocated chromosomes paired with an X during oogenesis to cause X nondisjunction and the production of a gamete lacking both X's but carrying the translocation.

Hauschka[5] has described a case that illustrates the third category. A father with XYX constitution produced among his progeny a triplo 21. It is proposed that the gamete giving rise to the mongoloid arose from pairing between the extra Y and chromosome 21 to produce a sperm carrying two chromosomes 21 and an X. A case reported by Therman *et al.*[6] involves D trisomy and Turner's syndrome in two sisters. If a heterozygous inversion were present in a pair of D chromosomes of the father, the association between the Y and D chromosomes with random assortment of the other two numbers could lead to the formation of both XDD and the OD sperm.

A number of reports describe the appearance of Turner's or Klinefelter's syndrome and mongolism in the same sibship. These types of aneuploidy

might be expected to be correlated among the progeny of fathers of a constitution characterized by irregular pairing of the sex chromosomes. The application of the distributive hypothesis to human gametogenesis would seem therefore to provide a reasonable genetic explanation for the correlated appearance of apparently unrelated abnormalities in pedigrees.

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[2]P. S. Moorhead *et al.*, *Am. J. Human Genet.* **3**, 32–46 (1961).

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2.9 SPONTANEOUS AND X-RAY-INDUCED EXCHANGE IN *DROSOPHILA* FEMALES

P. A. Roberts

The behavior of different regions of the X chromosome suggests a functional organization which transcends the cytological divisions of heterochromatin and euchromatin. For example, x irradiation of the normal X chromosome increases recombination in the centromeric heterochromatin and adjacent euchromatin but decreases recombination in more distal euchromatin. Also, recombination is increased in proximal regions moved distally and decreased in distal regions moved proximally by inversion. Investigations of these phenomena were carried out using normal and inverted X chromosomes.

Materials and Methods. – Inversion(1)sc^8 inverts almost the entire X chromosome, moving 90% of the centromeric heterochromatin to the tip of the X and changing the position of the euchromatic regions with respect to the centromere. Newly eclosed adult females, homozygous for the normal or inverted X chromosome but heterozygous for recessive markers, were exposed to 4000 r of x irradiation, mated, and transferred daily for 12 days. In this way, recombination was measured in brood fractions sampling chromosomes irradiated in different stages of meiosis and in oogonia. Some females carried heterozygous autosomal inversions which increase recombination between X chromosomes. Essentially the same procedure was followed for females which carried the above X chromosomes but were homozygous for the third chromosome mutant *c3G*, which suppresses spontaneous recombination. This made it possible to compare x-ray-induced oogonial recombination frequencies with the recombination frequencies which result when irradiated chromosomes go through meiosis.

Results and Discussion. – X-ray-induced exchange in the heterochromatic regions was significantly greater in early oocytes and oogonia than in late oocytes. Exchange in the 90% of the heterochromatin which was moved to the tip of the X was greater than the amount of exchange in the whole of the heterochromatin in its normal position. If, as has been shown in other organisms, exchange is dependent on proximity of broken ends at the time of irradiation, the heterochromatic regions of homologs are probably more closely associated in early oocytes at the time of crossing-over and in oogonia than in late oocytes. The increase in both spontaneous and x-ray-induced exchange in proximal regions which have been moved distally suggests that effective pairing of homologs is normally lowered in centromeric regions, perhaps as a compensation for heterochromatic associations in these regions. There is no evidence for persistent alterations of pairing between homologs resulting from the presence of heterozygous autosomal inversions.

The distal decrease in exchange observed when irradiated chromosomes undergo meiosis might be, in part, a result of homozygosis for distal loci caused by exchange in proximal heterochromatin. The crossover decrease in several euchromatic regions of the sc^8 homozygote (most of the induced exchange at the tip) is comparable to that in the normal X chromosome indicating that homozygosity is a minor contributing factor to the distal decrease. A comparison of x-ray-induced exchange in the normal and inverted chromosomes and in the same chromosomes in the presence of *c3G* indicates that most of the heterochromatic increase can be attributed to exchanges induced in oogonial chromosomes at the time of irradiation.

The proximal euchromatic increase and the euchromatic decrease apparently result from effects of x irradiation which persist through at least one cell division and alter the behavior of chromosomes during meiosis.

2.10 CROSSING-OVER IN CLARET-NONDISJUNCTIONAL *DROSOPHILA* FEMALES

D. G. Davis

Introduction. – In the course of previous studies of abnormal chromosome behavior associated with the mutant claret-nondisjunctional in *Drosophila* it became clear that nondisjunction always occurs at the first meiotic division.[1] Following passage of both dyads to one pole at anaphase I, segregation of monads from each dyad, and two-by-two assortment at anaphase II, the egg pronucleus may receive two X chromosomes. This theory of the ca^{nd} defect in egg maturation postulated that the high rate of nondisjunction observed was not a direct result of abnormal chromosome behavior per se but of abnormal spindle fiber formation or function. If, in fact, the chromosomes paired abnormally and nondisjunction were a result of defective bivalents, then nondisjunction might be accompanied by abnormal crossing-over. Therefore, the crosses described below were undertaken to test the validity of the general assumption of normal crossing-over in ca^{nd} females.

Results and Discussion. – Homozygous ca^{nd} females with heterozygous, multiply marked X chromosomes (y^2 *sc cv v f*·sc^{v1} y^+/y) were mated to males having a dominantly marked X (*B*), thus permitting recognition of the exceptional females as non-*B* carriers. Exceptional females so obtained were then backcrossed singly to *B* males, and their regular sons were examined to determine the X-chromosome genotypes of the exceptional female mothers. Regular male siblings of these exceptional females were controls. Table 2.10.1 shows that crossing-over in bivalents that subsequently nondisjoined did not differ appreciably from those that subsequently disjoined regularly. The data also fail to show any significant differences either in the frequencies of noncrossover strands recovered between exceptional females (0.511 ± 0.034) and regular males (0.463 ± 0.016), or in the coincidence values of double crossing-over.

Table 2.10.1. Crossing-Over in the X Chromosome of Regular and Nondisjunctional Eggs of ca^{nd} *Drosophila* Females

Region	Regular Males	Exceptional Females
sc - cv	10.4	10.7
cv - v	19.9	16.7
v - f	21.9	16.7
f - y^+	12.2	15.9

Although no analysis of tetrad exchange frequencies is possible at this preliminary stage, there is nothing to suggest unusually high frequencies of multiple exchanges in nondisjunctional eggs such as was reported recently[2] for normally occurring primary exceptions.

[1] D. G. Davis, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 58–59.

[2] J. R. Merriam and J. N. Frost, *Genetics* **49**, 109 (1964).

2.11 MUTANT TYPES AT THE NOTCH LOCUS IN *DROSOPHILA*

W. J. Welshons Elizabeth S. Von Halle
Bobbie J. Scandlyn

The Notch locus is a pseudoallelic system containing four arbitrarily defined classes of mutations: recessive visible mutations, dominant Notch wing mutations with recessive lethal effects, dominant bristle mutations with recessive lethal effects, and straight recessive lethal mutations having no discernible phenotype when heterozygous to a normal allele. All recessive lethal mutations, with or without a dominant phenotype, do exhibit pseudo-dominance when combined heterozygously with any of the recessive visibles, and, to date, all heterozygous combinations of recessive lethals fail to complement.

Many of the mutations at this locus have been mapped, and these recombination studies are continuing. Mapping is routinely carried out with respect to the four recessive visibles existing in the linear order *fa*, fa^{no}, *spl*, and *nd*. A new recessive lethal N^{55e11} has been localized to the left of *fa*, and a fifth recessive visible, nd^2, has been recently found. Females w^a nd/nd^2 *rb* crossed to w^a *nd rb* males produced two wild-type males in approximately 100,000 progeny, thus placing nd^2 to the right of *nd*. From recombination tests of N^{60g11} with nd^2 and *nd*, the tentative order of these three closely linked mutants is N^{60g11}, *nd*, and nd^2.

Classical studies have placed the locus of Notch in salivary band 3C7. Granting the validity of this conclusion, it follows that all the separable mutant sites demonstrated by pseudoallelic studies are localized to the same salivary band. One would therefore expect that the recessive lethal N^{264-39}, deficient for 3C7, would not recombine with any of the pseudoalleles. In practice, this deficiency $-N$ does recombine freely with *fa* and *spl* but fails to recombine with the two closely linked but separable mutants fa^{no} and N^{264-40} localized between *fa* and *spl*. From this combination of genetical and cytological evidence, one can conclude that only two of the known mutant sites fa^{no} and N^{264-40} could exist in band 3C7. Since there are many other mutant sites with positions to the left and right of these two it follows that the Notch locus cannot be confined to a single salivary band.

3. Cytochemistry and Cell Reproduction

R. C. von Borstel[a]
I. L. Cameron
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J. G. Carlson[b]
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F. M. Ritossa[c]
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3.1 H^3-THYMIDINE DERIVATIVE POOLS IN RELATION TO MACRONUCLEAR DNA SYNTHESIS IN *TETRAHYMENA PYRIFORMIS*

G. E. Stone O. L. Miller, Jr.[1]
D. M. Prescott[2]

Introduction. – Experiments with single *Tetrahymena*[3] have shown that DNA synthesis can be initiated in the absence of essential amino acids, but the DNA increase is limited to 20% (microspectrophotometry). For this increase, H^3-thymidine of the medium is not utilized. If cells are deprived of amino acids after DNA synthesis has begun, synthesis goes to completion and H^3-thymidine of the medium is readily incorporated into DNA. These results suggest that thymidine kinase (and probably other enzymes concerned directly with deoxynucleotide production) may normally be synthesized near the time DNA synthesis begins. During amino acid deprivation, however, thymidine kinase presumably cannot be produced and DNA synthesis is limited by the size of pre-existing deoxynucleotide pools left over from the previous round of DNA synthesis.

The possible presence of a continuing pool of thymidine derivatives has been directly investigated in *Tetrahymena* in two ways: by an autoradiographic procedure for detecting water-soluble substances in single cells[4,5] and by labeling DNA with H^3-thymidine and observing the dilution of label through two subsequent generations.

Results and Discussion. – *Autoradiography of Soluble Pools.* – Briefly the method consists of incubating cells in known stages of the cell cycle for short periods in H^3-thymidine. The cells are washed free of exogenous precursor, rapidly dried on a slide, then covered with either a dry film or a gelled but wet emulsion. In previous experiments, cells incubated during the macronuclear DNA synthesis period and immediately washed, dried, and covered with a dry film showed radioactivity confined to the nucleus. When similar

cells were covered with wet, gelled emulsion, a soluble radioactive component was extracted and deposited in the film over and around the cell.

In the present experiment, cells were incubated during the last hour of the S period and were in late S or early G1 when washed free of exogenous H^3-thymidine. After washing, the cells were returned to incubation pipets and allowed to continue growth in nonradioactive medium until the end of G2, when division furrows become evident (*ca.* 40 min). The results were similar to those obtained when cells were dried down immediately after incubation in the S period. When the labeled dividers were covered with dry film, radioactivity again was localized over the macronucleus. When covered with wet, gelled film, silver grains were present over the cytoplasm and around the cell. Unlabeled control cells were incubated (for at least one cell cycle) in the medium in which the labeled dividers were carried through G2. They showed no incorporation of label. These results show directly that a sizable intracellular pool of thymidine derivatives remains in this cell during a period when no DNA synthesis is going on.

Dilution of Incorporated Label. – To determine if the continuing pool of soluble thymidine derivatives is utilized for DNA synthesis in subsequent S periods, the following experiment was performed. Cells were incubated in H^3-thymidine from the beginning of the S period to the following division. The dividing cells were then removed from the incubation medium and thoroughly washed. Upon separation of the two daughter cells, one was dried on a slide and its sister allowed to go through another cell cycle which included another DNA synthetic period (in the absence of label). At the second division, both daughter cells were dried or in some instances one daughter cell was killed and its sister allowed to go through yet another cell cycle, whereupon the resulting daughter cells were dried on a slide. All cells of one line were dried on the same slide. The cells were extracted with acid-alcohol and covered with emulsion for exposure.

If no usable pool of thymidine derivatives exists from one synthetic period to another, grain counts should be reduced 50% at each subsequent division. If a continuing pool does exist, the daughter cells of the second division would be expected to show less than a 50% decrease while the products of the third division should halve the grains of the second division if the continuing pool goes through a complete turnover during the second DNA synthetic period. Comparison of grains over first-division cells vs second-division cells were obtained for 23 cell lines, while comparisons of second- and third-division cells were available for nine clones. The results of the experiment show that the total number of grains over the two second-division daughters was significantly higher than the number over the first-division daughter cell (P = 0.005–0.001), but that the number over the two third-division daughters was not significantly different from the number over the second-division cell (P = 0.80–0.70). Control cells present with labeled cells in the incubation pipets during first S period after labeling showed no incorporation of H^3-thymidine.

The combined results of the soluble-pool autoradiography and the label dilution experiment show that in *Tetrahymena pyriformis* a usable pool of soluble thymidine derivatives remains in the cell from one S period to the next but is incorporated into DNA within one or less S periods. It is of interest that the autoradiographic estimate of the size of the continuing pool was 24% of the incorporated thymidine, a value in close agreement with the 20% increase in DNA measured by microspectrophotometry in the amino acid deprivation study.

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[1]Now with Cell Growth and Differentiation Group.

[2]Present address: University of Colorado Medical Center, Denver.

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3.2 CYTOLOGICAL RESPONSES OF BROWN FAT TISSUE IN COLD-EXPOSED RATS

I. L. Cameron R. E. Smith[1]

Introduction. – The physiological significance of brown adipose tissue has remained relatively

obscure until its recent emergence as a thermogenic effector system in cold-exposed rats and in hibernating mammals during arousal. In view of findings with rats during cold acclimation that both mass and respiration of brown adipose tissue from all of the various sites appeared to increase, two main questions arose: first, does this increase in mass represent an increase in cell number, and if so, second, does the cytogenesis entailed simply represent mitotic activity of brown fat cells or a process involving cellular differentiations? Finally, it was surmised from earlier work that during the response to cold there should appear changes in the relative amount of white adipose associated with the brown adipose tissue; hence an attempt was made to assess this effect on the nature and time rates of change in the cytologic patterns of response to cold.

Methods. – A series of 21 control and cold-exposed rats were given an injection of H^3-thymidine 1 hr prior to being killed. In another experiment on the cytogenesis of brown fat cells, two rats were exposed to cold for 72 hr, injected with labeled thymidine, and then killed after another 24 and 96 hr. The tissues were removed and fixed for histology by a double embedding technique. Sections 5 μ thick were stained with PAS, hematoxylin and eosin, Masson's trichrome method, and thionin. Another set of PAS prestained material was autoradiographed by the coating technique, exposed for three weeks, developed, and then stained with hematoxylin. In appropriate material, cell size was measured by planimetry of uniformly projected photomicrographs and the incidence of H^3-labeled DNA counted directly in randomly selected microscopic fields. Another group of 40 young adult rats was used to determine the weight changes of epididymal white fat pad and also the brown fat from various body sites.

Findings. – In young adult laboratory rats exposed to cold (6°C), the brown adipose tissue undergoes time-dependent increases in cellularity, vascular supply, and total mass (2.5-fold increase). These changes are largely complete after 16 days in the cold and concurrent generally with the development of a thermoregulatory state not greatly dependent upon shivering. Histologically the brown fat changes from a tissue having both unilocular and multilocular fat cell types to one having almost exclusively the latter. During the first 6 to 12 hr in cold, the multilocular cells lose their lipid vacuoles and decrease in size, but these features are restored to normal by 24 hr. Cell proliferation, as estimated by the DNA synthetic index method (using tritiated thymidine autoradiography), appears in the reticuloendothelial cells of the brown fat at one day of cold exposure, becomes maximal at four days, and returns to the control level by 16 days. In animals injected with tritiated thymidine on the third day of cold exposure and then maintained for one or more additional days in the cold, autoradiographs indicate that new brown fat (multilocular) cells arise by cytogenesis from reticuloendothelial progenitor cells and not by proliferation of existing brown fat cells. Throughout this and subsequent periods, cells of the epididymal white adipose tissue slowly decrease in size and the weight of this tissue also decreases. Because a thermogenic role in cold acclimation has been established for the brown fat, the reported changes are regarded as adaptive responses to a cold environment.

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3.3 MACROMOLECULAR SYNTHESIS AND CELL DIVISION IN PYRIMIDINE-STARVED *TETRAHYMENA*

I. L. Cameron

Introduction. – This is a supplement to the last semiannual report, which dealt with macromolecular synthesis that occurs after replacement of required pyrimidines in pyrimidine-starved *Tetrahymena*. Another question to be answered was how do these cells finish two complete cell cycles, including replication of their large macronucleus, without exogenous pyrimidines? The second point was where in the cell cycle do pyrimidine-starved cells stall?

Methods. – The total Feulgen positive material was determined microspectrophotometrically on logarithmically growing G1 cells as well as on 72-hr pyrimidine-starved cells. RNA turnover was determined in the starved cells by giving them H^3-adenosine and then preparing autoradiographs for

inspection. To determine if RNA turnover could supply pyrimidine-deprived cells with pyrimidine bases necessary in DNA synthesis, a sample of logarithmically growing cells were given a 15-min pulse to H^3-uridine. The cells were washed free of exogenous H^3-uridine. Half of the cells were placed in complete medium; the other half were washed and resuspended in pyrimidineless medium. Cell proliferation was followed after resuspension of the cells. Each group of cells was sampled after threefold increase in cell number. The fixed cells were digested with ribonuclease, and autoradiographs prepared and analyzed.

Results and Discussion. – The amount of Feulgen positive material in 72-hr starved cells was *not* significantly different than in G1 log-phase cells. Therefore it appears that starved cells stall at a point prior to macronuclear DNA replication.

H^3-Adenosine was quickly incorporated into macromolecular material (presumably RNA) even after 72 hr of pyrimidine starvation. This suggests continuing RNA turnover.

The macronucleus of cells in complete medium is larger than in the pyrimidine-deprived cells. The cells treated with ribonuclease that were grown in complete medium to a threefold increase in cell number showed the average of 1.0 silver grain per macronucleus and only 1.5% of the macronuclei have 4 or more silver grains. The pyrimidine-starved cells which were also grown to a threefold increase in cell number, then treated with ribonuclease, showed an average of 6.2 silver grains per macronucleus and 82% have 4 or more silver grains. The conversion of labeled RNA via turnover to supply pyrimidine precursors of DNA synthesis is indicated from these results. It would therefore appear that endogenous pyrimidine reserves enable pyrimidine-deprived cells to complete one or more cell and nuclear replications, and then the cells stall in a state prior to another nuclear replication.

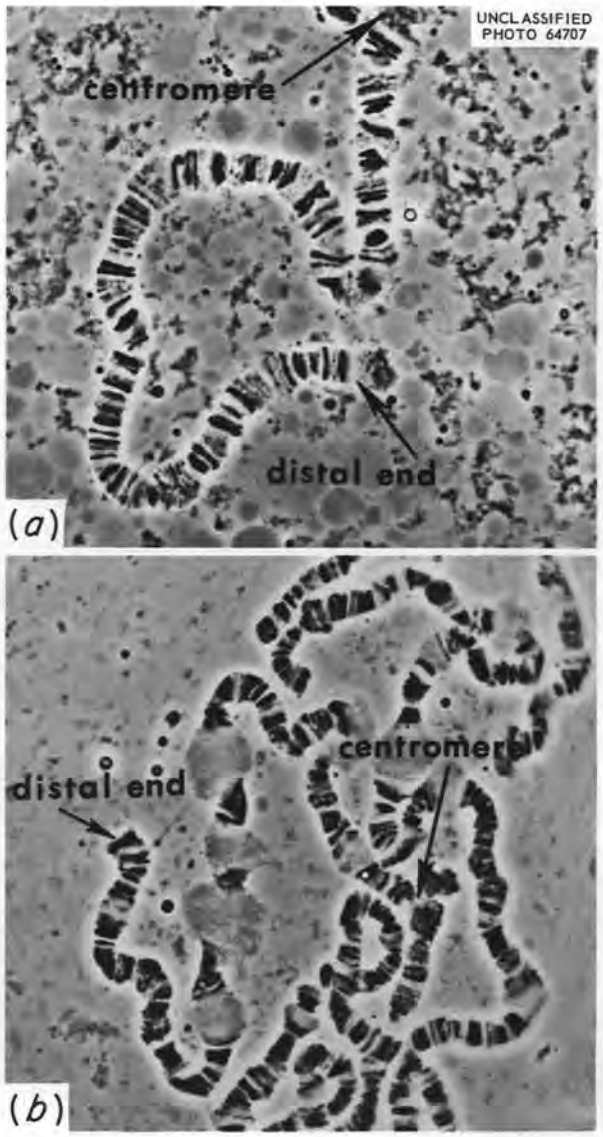

Fig. 3.4.1. (a) Left Arm of the Second Chromosome of *D. busckii*. The glands have been squashed in acetic acid 45% after 5 hr incubation in Ephrussi and Beadle solution. (b) The same arm of the second chromosome after 5 hr incubation in Ephrussi and Beadle solution containing 5 mg of ribonuclease per ml.

3.4 INDUCTION OF CHROMOSOME PUFFS IN *DROSOPHILA* WITH RIBONUCLEASE

F. M. Ritossa R. C. von Borstel

Introduction. – Chromosome puffs in dipteran salivary glands are structural modifications believed to be associated with gene action. Since RNA is synthesized in chromosome puffs, a series of experiments has been undertaken to attempt to elucidate puff structure and function in the presence of ribonuclease. This enzyme is known to enter cells, including salivary gland cells of *Drosophila*, and produce a number of alterations. We have found that ribonuclease can induce a large number of puffs.

Results. – Salivary glands of *Drosophila busckii* larvae reared at 25°C were excised and incubated in Ephrussi-Beadle solution[1] containing 3 to 5 mg/ml ribonuclease (Sigma 5X crystallized). For the control, glands were incubated in Ephrussi-Beadle solution only.

After about 1 hr of incubation, the nucleolus disappears almost completely – only a small remnant is found around the organizer. After 4 to 7 hr of incubation in ribonuclease, several puffs are induced. The efficiency of induction increases with the duration of the incubation time. After 7 hr the proportion of nuclei showing new puffs is 90% or more. At this time the chromosomes start to become more dense, and there is some nonspecific stickiness. There are about 40 puffs induced, several puffs being present in each arm of every chromosome; the puffs occur always in the same chromosomal regions and are generally larger than puffs that occur normally during larval development.

The puffs induced in the left arm of the second chromosome are shown in Fig. 3.4.1. Puffs are induced in regions 22A, 29B, 30B, 32C-D, 38A, and 39C. These positions are deduced from the chromosome map of Krivshenko.[2] The puffs at 30B, 31B, and 38A (previously called 2L14, 15, and 20) can also be induced by anaerobiosis, temperature shock, and uncouplers of oxidative phosphorylation,[3] and are in fact the only puffs induced in the genome by these agents. Not all the puffs appear simultaneously; there is a sequence of induction. On the left arm of the second chromosome the puffs appear in this order: 30B, 31B, and 38A; 32C-D; 29B and 39C; 22A.

[1] B. Ephrussi and G. V. Beadle, *Am. Naturalist* 70, 218 (1936).

[2] J. D. Krivshenko, *Genetics* 48, 1239 (1963).

[3] F. M. Ritossa, *Experientia* 18, 571 (1962).

3.5 ISOLATION AND PROPERTIES OF RIBOSOMES FROM *DROSOPHILA MELANOGASTER*

F. M. Ritossa Piero Cammarano[1]

Introduction. – Changes in puffing patterns are believed to represent differential activation of genes. It was thought that puff induction might be paralleled by changes of the ribosomal patterns at the cytoplasmic level; consequently, preliminary experiments were undertaken to isolate and characterize the ribosomes from larval stages of *Drosophila melanogaster*.

Results. – *Drosophila* larvae were homogenized in an all-glass apparatus with two volumes of a medium containing 0.05 *M* Tris pH 7.6, 0.025 *M* KCl, 0.005 *M* 2-mercaptoethanol; when present, Mg^{2+} was either 0.1 or 5 m*M*. The homogenate was centrifuged 20 min at 20,000 × *g* and the resulting postmitochondrial supernatant was further centrifuged at 105,000 × *g* for 90 min. The material sedimenting at 105,000 × *g* was resuspended in the homogenization medium and immediately used for analysis in a sucrose density gradient. At times sodium deoxycholate (1.2%) was added to the postmitochondrial supernatant, and in this case the material sedimented at 105,000 × *g* was resuspended in the above medium and recentrifuged at 105,000 × *g* for 90 min. Occasionally homogenization was performed by grinding the tissue under liquid nitrogen; the resulting powder was resuspended in the homogenization medium and processed as above. Isolation of the particles in the medium containing 5 m*M* Mg^{2+} led to the appearance of a heavy peak of approximately 170 S. Treatment of the isolated material with amounts of ribonuclease which are known to result in selective breakage of the interribosomal RNA (10 mμg/mg of RNA) in a variety of materials did not alter the sedimentation profile of this peak. The same sedimentation profile was observed when deoxycholate was used during the isolation procedure.

Centrifugation of the same preparations in a sucrose density gradient in the absence of Mg^{2+} results in the resolution of a minor protein component uniformly spread throughout the gradient and a sharp peak sedimenting in the region pertaining to particles of sedimentation constant 80 to 83; this peak showed a 280/260 ratio typical of ribonucleoprotein particles (0.54). No evidence existed for the appearance of subunits of the main peak component comparable to the 50-S and 30-S subunits described in other organisms. The extent of these phenomena was not influenced by either changes in the homogenization conditions or by the use of deoxycholate during the isolation procedure. In addition, replacement of magnesium with calcium ions at identical concentration did not alter the picture nor lead to

the appearance of polysomal peaks although the contrary has been reported.[2] In other experiments, the particles were isolated in the presence of 0.1 m*M* Mg and analyzed in sucrose density gradient of different magnesium concentrations. The increase of magnesium content in the sucrose gradient from 0.1 to 5 m*M* led to progressive aggregation of the 80-S particles to form the heavy sedimenting peak seen in particles isolated in the presence of 5 m*M* Mg, showing that partial aggregation of the monomeric particles occurred already in the presence of 0.5 m*M* Mg concentration. It may be noted that the 80-S component isolated from the corresponding region of the sucrose density gradient failed to reaggregate when subsequently centrifuged in a gradient containing 5 m*M* Mg.

Discussion. – The foregoing results indicate that in larvae of *D. melanogaster* isolation conditions similar to those extensively employed for isolation of polysomes in a variety of organisms lead to the appearance of only one heavy sedimenting peak, resulting from the nonspecific binding of individual ribosomes with a, so far, undefined protein component in the presence of high magnesium concentration.

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[1]Enzymology Group.

[2]M. Shurin, *J. Cell Biol.* 19, 10A (1963).

3.6 STUDIES OF AMINO ACID AND URIDINE INCORPORATION IN SALIVARY GLANDS TREATED WITH RIBONUCLEASE

F. M. Ritossa Hewson Swift[1]
R. C. von Borstel

Introduction. – It has been possible to show that chromosome puffs can be induced by ribonuclease.[2] Since it is known that, under normal conditions, puffs are active centers for RNA synthesis (and possibly a visible indication of active genes),[3] a series of experiments was undertaken to determine the turnover of RNA in those regions and the possible influence on protein synthesis of ribonuclease by use of tritium-labeled uridine and L-leucine.

In the course of the isotopic labeling experiments on the salivary glands of *Drosophila busckii*, two principal observations were made: (1) after ribonuclease action in vivo, a certain amount of uridine labeling still remains in the very active puffs and in the puffs induced by ribonuclease itself; and (2) ribonuclease inhibits amino acid incorporation into protein. Both of these actions show a degree of variability of expression which is reduced as incubation times are prolonged. We shall consider these observations separately.

Results. – The first observation is that of continued, though reduced, presence of uridine labeling in the puffs after ribonuclease action in vivo. As a control, puffs were induced by anaerobiosis and incubated with 0.1 mc/ml tritium-labeled uridine (3.7 c/m*M*, New England Nuclear Corporation) in Ephrussi-Beadle solution for 1 hr prior to fixation and subsequent coating with NTB3 emulsion. The induced puffs were treated under the same conditions except that incubation in vivo was done in the presence of 5 mg/ml ribonuclease (Sigma) in the solution. The uridine labeling under the conditions of this experiment disappears from the cytoplasm and the nucleolus except in the proximity of the nucleolus organizer.

After 5 hr of incubation many puffs are induced in the chromosomes of the salivary gland.[2] When labeled uridine was also placed in the incubation mixture, some label is found in all the puffs. The 5-hr control (without ribonuclease) shows the nucleolus and the chromosome arms to be clearly labeled. Where ribonuclease has been in the incubation mixture, the label is almost completely restricted to the puffed regions. This is also true when labeled uridine is added after the ribonuclease has acted for 5 hr and the ribonuclease-induced puffs are already present. The radioactivity can be attributed to the presence of ribonucleic acid since after fixation it is removable by ribonuclease treatment.

The ribonuclease-induced puffs show a metachromatic staining with azure B that is much more intense than when normal puffs are stained.

The second observation is that ribonuclease inhibits incorporation of the amino acids into proteins. This is demonstrated by the fact that cells incubated for 30 min with 0.1 mc/ml tritium-labeled L-leucine (0.5 c/m*M*, Schwarz) after an initial incubation for 2 hr in Ephrussi-Beadle solution show strong incorporation, but if cells were incubated in labeled leucine for 30 min after

an initial incubation for 2 or 5 hr in Ephrussi-Beadle solution with ribonuclease (5 mg/ml) no uptake of the leucine occurred.

Discussion. – Since it was possible to demonstrate less incorporated uridine in puffs induced by ribonuclease than in other puffs, but more RNA as demonstrated by the staining procedure, and we know that entry of uridine into the cell is not hindered by ribonuclease, we can conclude that there is a breakdown of RNA by ribonuclease which effectively dilutes the added labeled uridine.

From the observation of inhibition of amino acid incorporation by ribonuclease after a 2-hr treatment and the observation that puffs are induced by ribonuclease by 5 hr of treatment, it can be firmly concluded that the protein of the puffs pre-exists and is not formed during the process of puff production.

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[1] Zoology Department, University of Chicago.

[2] F. M. Ritossa and R. C. von Borstel, Sect. 3.4.

[3] W. Beermann, *Am. Zoologist* 3, 23 (1963).

3.7 SPONTANEOUS REVERSION RATES OF A SUPERSUPPRESSIBLE MUTANT DURING MITOSIS AND MEIOSIS

R. C. von Borstel Martha J. Bond
C. M. Steinberg[1]

Introduction. – For the spontaneous mutations thus far studied in yeast, reversions from auxotrophy to prototrophy occur at a rate 6 to 20 times higher during meiosis than during mitosis.[2] Further, it has been demonstrated that most of the meiotic reversions are associated with genetic exchange,[3] that is, recombination frequency increases strikingly between linked loci when a spontaneous mutation occurs between them. This suggests that these mutations are induced by unequal crossing-over, causing base-pair deletions or additions.

Approximately one-fourth of all mutant sites in every gene that have been induced by ultraviolet radiation are suppressible by supersuppressor genes,[4] here designated SS. A given SS allele can simultaneously suppress all loci. The currently favored hypothesis for the mechanism of action of SS genes requires that a suppressible mutant be of the base-pair substitution type and not of the addition-deletion type.[5]

A mutation which is of the base-pair substitution type should not revert by unequal crossing-over. Hence, the above two hypotheses predict that reversion rates for suppressible mutants should be nearly the same during mitosis and meiosis. A study of the reversion rates of such mutants would constitute a test of consistency of these two hypotheses.

Methods. – A supersuppressible mutant (tr_{5-2}) (kindly supplied by Dr. T. R. Manney) was chosen for the reversion experiment; another supersuppressible marker (ad_{2-1}) was also present in the stock. If the mutants selected for growth in the absence of tryptophan had reverted by way of the supersuppressor locus, then the revertants would also grow in the absence of adenine. If the reversions had proceeded by way of the tr_5 locus itself, then the revertants would still require supplementary adenine for growth.

The reversions occurring during mitosis were scored by use of the multicompartmented boxes of de Serres (unpublished). The growth medium was placed in 2-ml aliquots in each compartment; enough tryptophan was added so that the initial inoculum of 10^4 yeast cells per ml would multiply to about 5×10^6 cells/ml. If a reversion to tryptophan independence occurred, then the number of cells in that compartment would continue to multiply until 5×10^7 were present. Ten boxes (1000 compartments) were used in a typical experiment; the increased concentration of cells in a compartment containing a reversion is easily scorable. The mutations per nucleus per generation is determined from the fraction of compartments without a reversion using the P_0 term of the Poisson distribution.[6]

For reversions during meiosis, the cells were sporulated and spread on agar plates containing all growth requirements except tryptophan. Reversions occurring during meiosis were scored as colonies growing on the plate. The method for computing the reversion rates during meiosis has been given elsewhere.[2]

Results. – The reversions per nucleus per generation for the supersuppressible mutant tr_{5-2} were as follows: during mitosis, 1.47×10^{-8}; during meiosis, 2.60×10^{-7}. It can be seen that the reversion rate is approximately 20 times higher during meiosis. However, of the 515 colonies reverting during mitosis, none reverted by way of

SS genes. Of the 305 mutations occurring during meiosis, 289 reverted through SS genes as measured by simultaneous reversions to adenine independence, and 16 reverted for tryptophan independence only. Therefore, reversions at the tr_5 locus itself arose spontaneously at a rate of 1.37×10^{-8} mutations per nucleus per generation at meiosis, approximately the same rate as during mitosis.

Conclusion. – The data show no difference between mutation rates of a supersuppressible mutant (tr_{5-2}) during mitosis and meiosis. These data support the hypothesis that the mutant under investigation is a mutation of the base-pair substitution type.

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[1]Cytology and Genetics Group.

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[3]G. E. Magni, *Proc. Natl. Acad. Sci. U.S.* **50**, 975 (1963).

[4]D. C. Hawthorne and R. K. Mortimer, *Genetics* **48**, 617 (1963).

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[6]S. E. Luria and M. Delbrück, *Genetics* **28**, 491 (1943).

3.8 BREAKAGE OF LAMPBRUSH CHROMOSOMES INDUCED BY X RADIATION

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R. C. von Borstel

Introduction. – It is generally believed that the primary biological damage from radiation is mediated through effects in the cell nucleus and that the principal target is chromosomal DNA. The meiotic lampbrush chromosomes of certain amphibian oocytes can be isolated from the rest of the cellular components in a state which resembles their appearance in the living condition. They also have been described more precisely than any other chromosomes both morphologically and chemically. These chromosomes, then, would appear to be the best currently available for studying chromosome breakage at a level approaching the molecular. For example, this is the only cell type in which chromosome breaks can be seen immediately after irradiation without intervening changes in the cell cycle.

The first studies on radiation-induced breakage of lampbrush chromosomes were done by Duryee;[2] he came to the conclusion that much of the nuclear damage was mediated through effects on the cytoplasm.

Methods and Results. – When lampbrush chromosomes of *Triturus pyrrhogaster* or *Triturus viridescens* are isolated manually, mechanical breakage is common. In order to prevent this accidental breakage (since chromosome breakage was to be our criterion of radiation damage), the following technique was developed. Manually isolated nuclei are placed in a pepsin solution (1 mg/ml, pH 2). At this acidity, the nuclear envelope dissolves, allowing the chromosome set in control preparations to spread unbroken. The pepsin causes a general contraction and increased refractiveness of the chromosomes which aid in analysis of x-irradiated preparations. Manual isolations of chromosomes, executed extremely carefully, were done as controls after x irradiation to establish that pepsin isolations give an accurate estimate of the number of breaks.

The assay being used in this study of chromosome breakage is the frequency of free chromosome fragments per oocyte. Over the dose range used, almost all of such fragments result from single breaks terminal to chiasmata rather than proximal double breaks.

Dose-action data are presented in Table 3.8.1. By the assay used, *T. pyrrhogaster* appears more sensitive to radiation than *T. viridescens*. This probably is explained by three factors. *T. pyrrhogaster* has 12 chromosome pairs and *T. viridescens* has 11; thus the former has four more free ends. In *T. viridescens*, chiasmata, on the average, occur closer to the chromosome ends. Also in *T. viridescens*, homologous terminal associations, which are similar to chiasmata in limiting terminal deletions, are more frequent. Since the last factor is quite variable from animal to animal in *T. viridescens*, control and experimental oocytes for any one experiment must be obtained from the same animal.

From the data in Table 3.8.1, it is obvious that the same frequency of chromosome fragments is induced whether the chromosomes are irradiated when within intact oocytes, within intact isolated nuclei, or when free in the isolating medium.

Discussion. – Under the conditions of the experiment, chromosome breakage induced by x radiation is not dependent on the presence of any cytoplasmic

Table 3.8.1. Average Number of Chromosome Fragments per Oocyte Found Following X Irradiation of Lampbrush Chromosomes in Vitro and in Situ

Dose (kilorads)	Average Number of Chromosome Fragments per Oocyte		
	Whole Oocytes	Isolated Nuclei	Isolated Chromosomes
		T. pyrrhogaster[a]	
5	1.10 (48)[b]		
10	3.93 (105)	3.63 (32)	
15	5.21 (42)		
20	7.44 (18)		
		T. viridescens[c]	
5	0.63 (19)		
10	1.30 (20)		
15	2.40 (20)		
5	0.50 (10)		
10	0.80 (20)		
15	1.55 (20)		
20	2.15 (20)		
15	0.82 (11)		0.67 (9)
30	5.80 (10)		5.10 (10)
45	2.50 (12)		2.67 (12)

[a]Combined data on oocytes excised from ten animals.

[b]The number of preparations involved in each point is indicated in parentheses.

[c]Each experiment limited to oocytes obtained from a single animal.

component. In addition, nucleoplasm appears to play little, if any, role in x-radiation breakage since isolated chromosomes can be readily broken after considerable dispersal of the nucleoplasm.

With the exception of axial breaks, x radiation produces no immediately perceptible change in the morphology of isolated lampbrush chromosomes. When these chromosomes are treated with proteases or ribonuclease, a characteristic deformation occurs due to removal of a ribonucleoprotein matrix, but chromosome axes are not broken.[3] When the chromosomes are treated with deoxyribonuclease, however, breaks in the axes appear, but no changes occur in the matrix material. The close similarity between the effect of x radiation and the action of an enzyme which specifically breaks DNA suggests that the primary damage of x radiation may occur in a DNA component which maintains at least part of the linear continuity of the chromosome.

[1]Now with cell Growth and Differentiation Group.

[2]W. R. Duryee, *J. Natl. Cancer Inst.* **10**, 735–95 (1949).

[3]H. C. Macgregor and H. G. Callan, *Quart. J. Microscop. Sci.* **103**, 173–203 (1962).

4. Cell Growth and Differentiation

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4.1 COMPARISON OF THE INHIBITORY EFFECTS OF ACTINOMYCIN AND X IRRADIATION ON THE LENS REGENERATING SYSTEM

Tuneo Yamada P. J. Kohonen Marion Roesel

Earlier data showed that during lens regeneration of adult *Triturus viridescens,* cells go through changes in susceptibility toward Actinomycin.[1] Later, immunofluorescence data[2] suggested the possibility that the cells that are relatively resistant to Actinomycin coincide with cells that are beginning to synthesize lens antigens in their cytoplasm. Autoradiographic data (see Sect. 4.6) further indicated that the relative resistance may be correlated with cessation of DNA synthesis. Since these correlations may be of importance for further analysis of cellular differentiation, efforts are being made to collect more information on susceptibility and resistance of differentiative processes toward various inhibitory agents.

Control Series. – Two hundred twenty regenerates at various days from lens removal were studied in serial histological sections and classified into morphological stages of lens regeneration, according to Stone and Steinitz.[3] With these data a diagram was constructed which indicates the normal range of regeneration stages during the 12th to 28th days after lens removal.

Treatment with Actinomycin-D. – One microgram of the antibiotic per gram of body weight was injected daily into the body cavity of the lentectomized adult *T. viridescens.* A complete arrest of lens regeneration was observed when the treatment was continued for 10 to 11 days after lens removal. When four daily injections were made during various phases of regeneration, and the regenerates were studied 1 to 12 days later in serial sections, the data summarized in Table 4.1.1 were obtained. The main effects observed were (1) delay in the progress of regeneration without morphological abnormalities; and (2) abnormal lenses, in which the lens epithelium was degenerative, being disaggregated into separate cells and pycnotic, while the lens fiber cells showed relatively normal cellular differentiation, as expressed in increase in amount and acidophilia

Table 4.1.1. Lens Regeneration as Affected by Actinomycin Administered at Various Stages of Regeneration

Period of Treatment (days after lens removal)	Number and Type of Regenerates			
	Simple Delay	Abnormal Regenerates	No Effect	Total Number
3–6	17	0	0	17
8–11	13	0	5	18
10–13	18	15	6	39
11–14	10	4	1	15
12–15	0	9	0	9
13–16	1	7	0	8
15–18	2	37	1	40
16–19	2	8	1	11
34–37	0	8	0	8
42–45	0	5	0	5
	63	93	14	170

of cytoplasm and in decrease in nuclear basophilia (Fig. 4.1.1). An early treatment tended to produce a simple delay, whereas a later treatment frequently caused the abnormal lenses. In the normal iris and lens, no sign of damage was observed after the same treatment.

X Irradiation. – Total-body irradiation (200 r) of lentectomized animals did not affect lens regeneration; 1000 r produced effects which were similar to those obtained with the Actinomycin treatment. When irradiated on the 6th day after lens removal, the regenerate stopped developing at the depigmentation stage. Irradiation on the 12th or 16th day after lens removal led to formation of abnormal lenses which have morphological features closely resembling those obtained with Actinomycin.

Discussion. – The results of the Actinomycin treatment suggest that RNA synthesis dependent upon DNA is one of the factors controlling the progress of regeneration in its early phase. This is in harmony with the speculation that the en-

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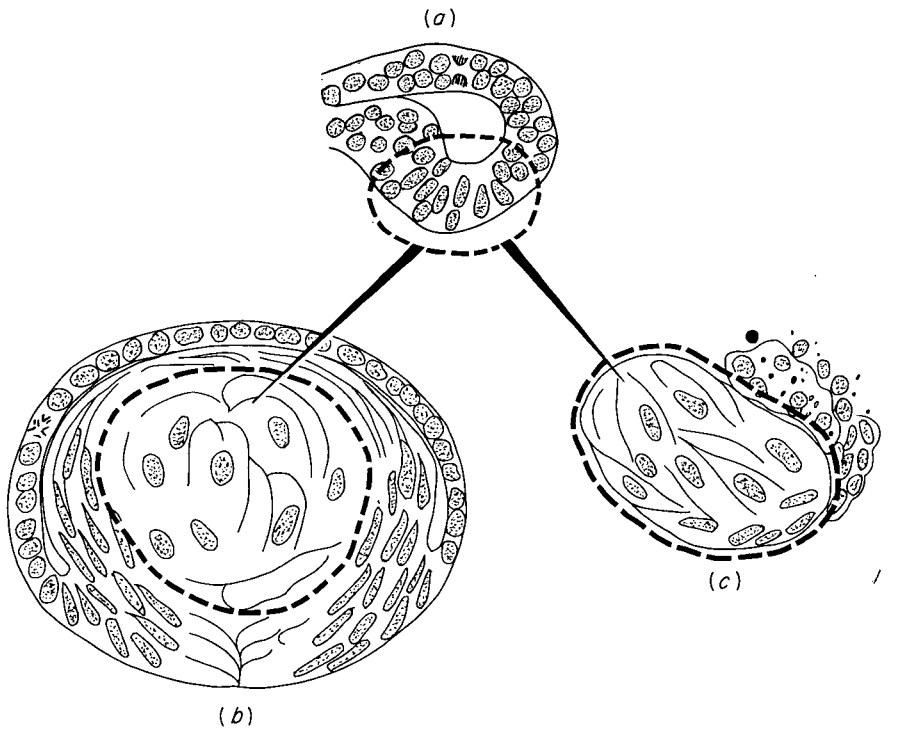

Fig. 4.1.1. Effects of Actinomycin on the Regenerating Lens. (*a*) Normal regenerate at stage V; (*b*) normal regenerate at stage XI, a control for (c); and (c) regenerate after treatment with Actinomycin. Circles in diagrams indicate the approximate boundary of the primary lens fibers.

hancement of RNA synthesis observed in the iris cell nuclei is the first basic cellular change leading to lens regeneration and involves a change in transcription of genetic information. The abnormal lenses observed after the Actinomycin treatment indicate that the cells of the lens epithelium or their precursor cells are highly susceptible to the treatment, while the prospective primary lens fiber cells can grow and differentiate in spite of the same treatment (Fig. 4.1.1). The basic parallel between Actinomycin effects and irradiation effects strengthens the idea that the activity of the nucleus is involved in the observed inhibitions. Cells that are susceptible to the treatments coincide with cells having a high level of RNA synthesis as observed by autoradiography. This would mean that differentiation of the lens fiber cells occurs relatively independently of new synthesis of RNA in the nucleus compared with other cells of the regenerate.

It is very likely that differential susceptibilities of the type suggested in this work also occur among various cell populations that constitute a developing embryo and are the basis for teratogenesis caused by various chemicals. The line of investigations adopted in our group for the analysis of lens differentiation may be useful for analysis of the mechanism of teratogenesis.

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[2]C. Takata, J. F. Albright, and T. Yamada, "Lens Antigens in a Lens-Regenerating System Studied by the Immunofluorescent Technique," to be published in *Developmental Biology*.

[3]L. S. Stone and H. Steinitz, *J. Exptl. Zool.* **124**, 469 (1953).

4.2 IMMUNOFLUORESCENCE STUDY OF LOCALIZATION AND APPEARANCE OF SPECIFIC FRACTIONS OF LENS PROTEINS IN TRANSFORMATION OF ADULT NEWT IRIS INTO THE LENS

Chinami Takata J. F. Albright[1]
Tuneo Yamada

Introduction. – It was shown in previous experiments[2] that in the lens regenerating system lens antigens first appear in the cytoplasm of a few cells of the inner layer of the regenerate on the 13th day after lens removal of the adult newt. Subsequently, fluorescence was found clearly localized in the cytoplasm of all lens fiber cells. Cells of lens epithelium also indicated lens antigens at later stages. The present work was done to study the mode of appearance and localization of each of the major components of lens proteins during lens regeneration in relation to the problem of cellular differentiation.

Materials and Methods. – To isolate lens proteins, the lens homogenate in 0.05 *M* Tris (pH 7.2) of the adult *Triturus viridescens* was centrifuged at 12,000 × *g* for 60 min at 1 to 2°C. The supernatant was applied to the Sephadex G-75 column (2 × 60 cm) in order to separate gamma crystallin from lens proteins according to the method of Bjork.[3] Measurement of absorbancy at 280 mμ indicated two peaks (Fig. 4.2.1). The eluted fractions corresponding to each peak were collected and concentrated to 7–10 mg of protein per milliliter by use of Carbowax 20 M. The first eluted peak (*A* + *B*) was filtered through the Sephadex G-200 column (2 × 50 cm). The eluted fractions belonging to each of two peaks were collected and concentrated, as described above.

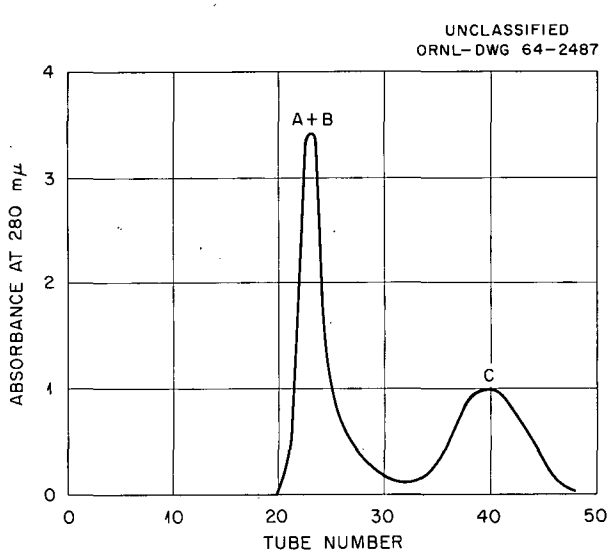

Fig. 4.2.1. Gel Filtration of Soluble Lens Proteins on Sephadex G-75 at pH 7.2. 1-ml sample; protein concentration, 32 mg/ml.

Antiserum to each of three peaks was obtained from one rabbit each. The gamma-globulin fractions from each antiserum were conjugated with fluorescein isothiocyanate and absorbed with mouse powder.[2] Regenerates of various stages were used as sources for antigens, and the histological procedures for immunofluorescence suggested by Sainte-Marie[4] were followed. All lens protein fractions and all antisera were examined by immunoelectrophoresis for their specificity.

Results and Discussion. – Three fractions, arbitrarily denoted *A*, *B*, and *C*, were obtained by gel filtrations, as shown in Figs. 4.2.1 and 4.2.2. Immunoelectrophoresis was carried out with the whole lens extract, or each fraction, in combination with the antiserum to the whole lens, with the antiserum to each fraction, or with the normal rabbit serum. As shown in Table 4.2.1, the immunoelectrophoretic patterns show that fraction *C* is free from alpha and beta crystallins and produces two to three precipitin lines belonging to the gamma-crystallin region. On the other hand, the antiserum to fraction *A* reacts not only with alpha crystallin but also with beta and gamma crystallins. The antiserum to fraction *B* does not show precipitin lines in the alpha-crystallin region, but does show four precipitin lines in the beta-crystallin region and two faint lines in the gamma-crystallin region. Therefore, to detect localization of alpha or beta crystallin, tissue sections were first flooded with the antiserum to fraction *B* or *C* and then stained with the fluorescein antiserum against fraction *A* or *B*. Gamma-crystallin was detected by simple application of fluorescent antiserum against fraction *C*.

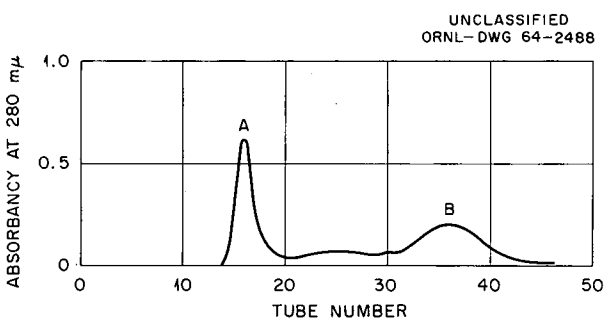

Fig. 4.2.2. Gel Filtration of Concentrated Fraction *A* + *B* on Sephadex G-200 at pH 7.2.

Each of three major groups of lens proteins (alpha, beta, and gamma crystallins) was observed in the cytoplasm of some cells of the inner layer of regenerates at Sato stage IV on the 13th to the 15th day after lens removal. The gamma crystallin was detected in the cytoplasm of all lens fiber cells. In 25- and 30-day regenerates the immunofluorescence for alpha and beta crystallins was positive in the cytoplasm of epithelial cells. However gamma crystallin was not detected in the same cells.

Table 4.2.1. Results of Immunoelectrophoresis

Antiserum	Antigen	Number of Precipitin Lines[a]		
		Alpha-Crystallin Region	Beta-Crystallin Region	Gamma-Crystallin Region
Whole lens	Whole lens	1 or 2	4	3
	Fraction *A*	1 or 2	1 and (1)	0
	Fraction *B*	0	4	0
	Fraction *C*	0	0	2
Fraction *A*	Whole lens	1 or 2	3	0 or (1)
Fraction *B*	Whole lens	0	4	0 or (2)
Fraction *C*	Whole lens	0	0	2 or 3

[a]Numbers in parentheses represent very faint lines.

The data indicate that cells of regenerates have specific combinations of three major groups of lens-specific proteins according to the regeneration stage and localization within the regenerate.

[1]Radiation Immunology Group.

[2]C. Takata, J. F. Albright, and T. Yamada, "Lens Antigens in a Lens-Regenerating System Studied by the Immunofluorescent Technique," to be published in *Developmental Biology*.

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4.3 CHROMATOGRAPHIC SYSTEM FOR THE COMPONENTS OF VERTEBRATE YOLK GRANULES

R. A. Wallace

The granules of the egg yolk[1] of the hen and the yolk platelets of the frog egg[2] have been shown to be homologous structures [i.e., they both contain almost exclusively a large lipophosphoprotein (lipovitellin, mol. wt = 400,000 in neutral solvents, protein P = 0.2–0.6%) and a small, highly phosphorylated phosphoprotein (phosvitin, mol. wt = 36,000, P = 8–10%) in the approximate molecular ratio of 1:2]. The relative insolubility of the lipovitellin (Lv) component in the usual solvent systems, however, has made it difficult to develop a general method for the isolation and purification of the yolk granule proteins. In continuing the previous work toward development of a separation method for the granule proteins using various adsorptive materials,[1,3–5] an analytical chromatographic procedure with the following characteristics has now been elaborated:

1. Single or multiple chromatographic columns can be repeatedly used, providing reproducible results.
2. A phosphate-free buffer system is employed throughout all operations (to ensure the reliability of phosphorus analysis performed on eluted proteins).
3. The granule proteins are completely adsorbed by the bed material and are completely (97% average recovery) eluted as relatively symmetrical peaks by a single gradient system.
4. The method is applicable to hen's egg yolk granules, to yolk platelets from the eggs of *Rana pipiens*, and presumably to other vertebrate yolk granules as well.

The chromatographic system finally developed (which can be scaled to any size) consists of a 32- by 1.9-cm (90-ml bed volume) column of TEAE-cellulose eluted with a concave gradient produced by a three-chambered gradient apparatus[6] (1000 ml total volume). The first two chambers each contain 335 ml of starting buffer [0.01 *M* citric acid–0.06 *M* 2-amino-2-methyl-1-propanol (AMP), pH 9.9], and the third chamber contains 330 ml of limit buffer (0.25 *M* citric acid–0.75 *M* AMP, pH 7.6). The maximum sample which can be used with this system is 250 mg. The capacity of the column for avian granule proteins can be increased 50% by use of 0.01 *M* citric acid–0.04 *M* AMP (pH 9.4) as the starting buffer, but the frog platelet proteins and the isolated Lv are barely soluble in this solvent. Conditions have been developed also for the preparation and dissolution of the granule proteins in the starting buffer, for the maintenance of columns for repeated use by appropriate packing and regenerative washing procedures, and for the nondestructive concentration of protein fractions by the refinement of a multiple ultrafiltration device.

Elution patterns obtained with chicken and frog yolk granule preparations are provided in Fig. 4.3.1. The granules from hen's egg yolk typically contain two lipovitellins (α-Lv and β-Lv), while the amphibian platelets contain only one lipovitellin (amph-Lv). A small amount of material designated as "α_2-Lv" is apparently derived from α-Lv since more is formed when isolated α-Lv is rechromatographed. The phosvitin peaks from both chromatograms contain phosvitin contaminated to varying extents (10–30%) with Lv. The amphibian phosvitin is the least contaminated and also absorbs rather poorly in the ultraviolet,[2] both factors contributing to the much diminished peak. For identification, all isolated samples were analyzed chemically for lipid and protein-phosphorus content as well as in the ultracentrifuge for homogeneity and dissociative behavior in alkaline solvents. The data, some of which are summarized for the Lv in Table 4.3.1, are in general agreement with previous results and indicate that the protein-phosphorus values found for the Lv are real and not due to a nonspecific binding of phosphate ions

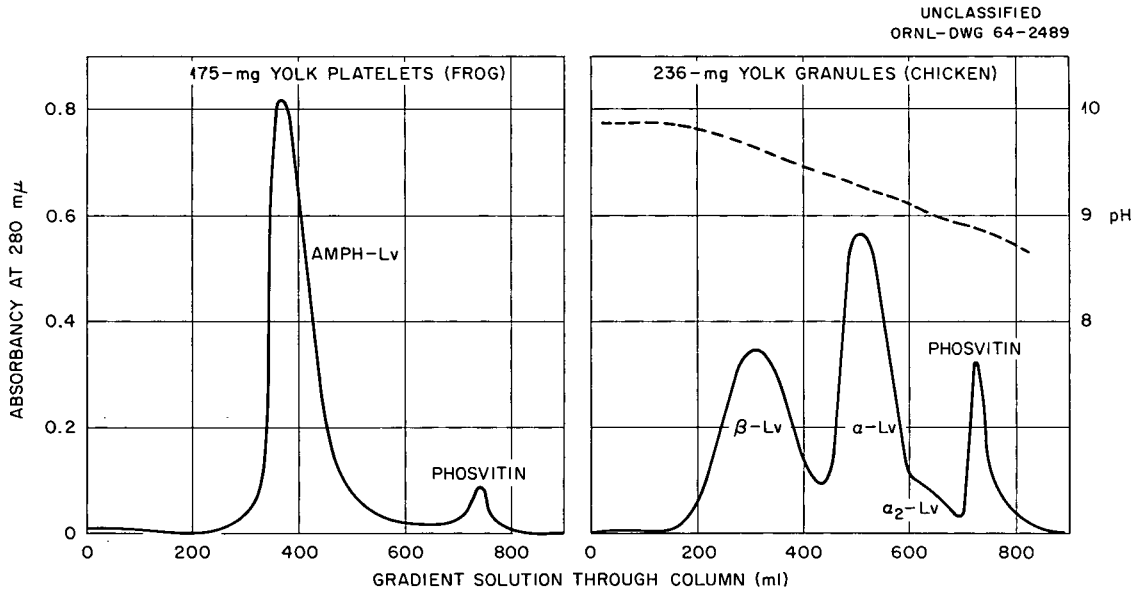

Fig. 4.3.1. Comparison of Chromatograms Obtained for Amphibian Yolk Platelets and Avian Yolk Granules Using TEAE-Cellulose and 1000-ml Gradient.

Table 4.3.1. Comparative Analytical Data for the Isolated Lipovitellins

Component	Protein-Phosphorus (%)	Lipid (%)	Percent Dissociated at pH 10.5
Avian β-Lv	0.24	17.9	92
Avian α-Lv	0.49	17.6	47
Avian α_2-Lv	0.48		49
Amph-Lv	0.37	17.7	14

from the bed material (hydroxyapatite[1,3,4]) or solvent.[5]

The elution positions for the various yolk granule components are provided in Table 4.3.2, which represents data accumulated from 40 separate chromatograms obtained with four different columns repeatedly used. The reproducibility appears good, and the isolated components rechromatograph as single entities (including the phosvitin with its Lv contaminant). A puzzling phenomenon, however, is the general tendency of the isolated Lv to be eluted at later stages upon rechromatography. This seems similar to what has been recently noted for serum albumin and attributed to an unspecified effect of lipidlike molecules.[7]

I feel that the methodology for the description of vertebrate yolk granules by a chromatographic procedure has been developed sufficiently to meet a broad application to various vertebrate forms – both for analytical and preparative purposes. Comparative studies of both crystalline (amphibian) and noncrystalline (avian) yolk granules will now be undertaken with a view toward phylogenetic relationships and the transfer of precursor serum proteins into the granular proteins of the oocyte during vitellogenesis.

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[7]E. A. Peterson and E. A. Chiazze, *Arch. Biochem. Biophys.* **99**, 136 (1962).

Table 4.3.2. Elution Position of Yolk Granule Proteins Under Various Conditions[a]

	Initial (Whole Granule) Chromatography		Rechromatography
	pH 9.4[b]	pH 9.9[c]	pH 9.9[c]
Avian β-Lv	(10) 366 ± 4	(6) 310 ± 4	(6) 325 ± 15
Avian α-Lv	(10) 546 ± 8	(6) 508 ± 10	(6) 534 ± 3
Avian phosvitin	(10) 765 ± 9	(6) 727 ± 11	(2) 733
Amphibian Lv	(2) 470	(6) 378 ± 7	(2) 400
Amphibian phosvitin	(2) 785	(5) 728 ± 9	

[a]The elution position of each peak is given in terms of milliliters of gradient *off* column. Figures in parentheses indicate number of chromatograms.

[b]Starting buffer was 0.01 *M* citric acid–0.04 *M* AMP.

[c]Starting buffer was 0.01 *M* citric acid–0.06 *M* AMP.

4.4 ELECTRON MICROSCOPIC STUDIES ON FORMATION OF THE NUCLEOLUS DURING AMPHIBIAN EMBRYOGENESIS

Shuichi Karasaki

Introduction. – During the development of the amphibian embryo, the nucleolus makes its first appearance in the cell at the time of gastrulation and gradually assumes its typical structure.[1] Attempts were made to study the origin and structural elaboration of the nucleolus during early embryogenesis and to relate the data with those on the site of uridine incorporation as revealed with electron microscopic autoradiography.

Methods. – Small pieces of the ectodermal and mesodermal regions were isolated from embryos of *Triturus pyrrhogaster* at various stages and treated for 3 hr in a medium with 50 μc/ml of tritiated uridine. After different periods of immersion in a nonradioactive culture medium, the explants were fixed in an OsO_4 solution and embedded in Epon. Grids containing thin sections were coated with a film of nuclear track emulsion and exposed for two to four weeks.[2] After development, they were treated with an alkaline lead stain.

Results and Discussion. – In embryonic cells of the cleavage and blastula stages, the interphase nucleus lacks a nucleolus. The only structural component observable with the electron microscope is dispersed chromatin fibrils about 100 A in diameter. At the gastrula stage the primary nucleoli originate within strands of the chromatin material as small, dense bodies consisting of fibrous material of about 40 A diameter. An enlargement of the nucleolus occurring during successive developmental stages involves the acquisition of a granular component of 150 A diameter, forming a cortical layer around the dense fibrous core. This new component closely resembles cytoplasmic ribosomes in size and density. At the same time, one begins to see larger particles of 300–500 A diameter intermingled with the chromatin fibrils. At the tail-bud stage or later, these particles fill the interchromatin spaces.

Autoradiographs of gastrular cells show silver grains exclusively over the nucleus, whereas in differentiating cells at the tail-bud stage or later, some fraction of the silver grains are over the cytoplasm. Within gastrular cells, both primary

nucleolus and chromatin strands were labeled; however, there were always more silver grains per area in the former than in the latter. In the experiments applied to cells at the tail-bud stage or later, labeled material is first localized in the fibrous core of the nucleolus and in the chromatin strands. After a longer culture in nonradioactive medium, labeled materials also appear in the granular cortex of the nucleolus and in the interchromatin spaces. These findings may suggest that the fibrous core is involved in formation of the granular component.

[1]S. Karasaki, *Embryologia* 4, 273 (1959).

[2]O. L. Miller, Jr., and A. Rebecca Stevens, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 71.

4.5 EFFECT OF ACTINOMYCIN-D ON DEVELOPMENT OF AMPHIBIAN EMBRYO

Sara Eisenberg-Wieder P. J. Kohonen
S. K. Brahma

Introduction. – Many reports indicate that RNA synthesis is minimal during cleavage in the amphibian embryo and increases during gastrulation and neurulation.[1] However, it is not known to what extent the progress of early development of embryos is dependent upon RNA synthesized during development. This study was undertaken in order to determine if some normal events of embryonic development could be suppressed by Actinomycin-D, which has been reported to inhibit DNA-dependent RNA synthesis.[2]

Materials and Methods. – Experiments were conducted using embryos of *Xenopus laevis*. Embryos at early cleavage, blastula, and gastrula stages with intact vitelline membrane were placed in Actinomycin-D solutions ranging in concentration from 10, 20, 40, to 80 μg/ml. Controls at a similar series of developmental stages were used.

Results. – In a series of various concentrations of Actinomycin-D, a strong inhibitory effect was observed at 40 and at 80 μg/ml, and a detectable one at 20 μg/ml. A concentration of 10 μg/ml had no effect.

Table 4.5.1. Effect of 24-hr Treatment with Actinomycin-D (40 μg/ml) on the Development of *Xenopus laevis* Embryos

	Stage at Beginning of Experiment		
	Early Cleavage	Blastula	Early Gastrula
Experimental Group			
Number with arrested development	50[a]	3	3
Number with normal development	11[b]	10[c]	19[c]
Total number of embryos	61	13	22
Control Group			
Number with arrested development	2	1	2
Number with normal development	18[b]	11[c]	16[c]
Total number of embryos	20	12	18

[a]Early gastrulae.
[b]Late neurulae.
[c]Tail-bud embryos.

After a 24-hr treatment with effective concentrations of Actinomycin-D, inhibitory effects were observed in the embryos treated at early cleavage stages (Table 4.5.1), where this antimetabolite caused development to stop at the gastrula stage, resulting in exogastrulae. When blastulae and gastrulae were treated similarly, development was not affected in the majority of cases after a 24-hr treatment; however, delayed effects were observed in disturbance of hatching and death of the embryos at the hatching stage.

Discussion. – The results indicate that morphogenetic movements connected with gastrulation are more sensitive to the treatment with Actinomycin-D than cleavage and organogenesis occurring

in neurulae and tail-bud embryos. Efforts are being made to avoid the possible permeability barrier by the tissue-culture technique.

Further data suggest the possibility that, under certain conditions, the antibiotic can suppress neural induction.

[1]J. Brachet, *The Biochemistry of Development*, Pergamon, New York, 1960.

[2]E. Reich, *Cancer Res.* **23**, 1428 (1963).

4.6 LOCALIZATION OF DNA SYNTHETIC ACTIVITY IN THE LENS-REGENERATING SYSTEM

Sara Eisenberg-Wieder Tuneo Yamada

Introduction. – Several autoradiographic studies using H^3-uridine and H^3-leucine have shown that in the common newt, *Triturus viridescens*, removal of the lens is followed by an increase in RNA and protein synthesis in the iris which later regenerates a lens.[1,2] The present investigation attempts to relate DNA synthesis to RNA and protein synthesis in this regenerating system.

Materials and Methods. – Adult newts were lentectomized and injected with H^3-thymidine (1 μc per gram of body weight) at 3, 5, 7, 10, 15, 25, and 30 days after lens removal. Twelve hours after injection the animals were killed, and autoradiographs of lentectomized eyes were prepared. The percentage of labeled cells and mitotic figures in relation to the number of cells present in the dorsal iris, regenerate, and ventral iris was measured.

Results. – DNA synthesis in the cells all over the dorsal marginal iris begins to be activated at 5 days after lens removal (stage I). The percentage of labeled cells is about 25% at 5 days and gradually increases to a maximum at 11 days (stage IV), when about 28% of the cells are labeled. At 15 days after lens removal (stage VII), cells starting to undergo fiber differentiation stop synthesizing DNA. From this stage on, the DNA synthetic activity decreases and becomes limited to the lens epithelium in the older regenerates (stages X and XI; 25 to 30 days after lens removal). The mitotic activity appears in the 7-day regenerates (stage II) and is most frequent at 15 days, when about 21% of the cells are in mitosis.

In the ventral iris, DNA synthesis is activated and reaches its maximum value at 5 days after lens removal. This value is higher than that of the dorsal iris. The percentage of labeled cells then decreases sharply, so that at stage IV it is significantly lower than that of the dorsal iris. The mitotic activity reaches its maximum at 15 days after lens removal, but it is always lower than that in the dorsal iris.

Discussion. – This study shows that lens removal causes an increase in the DNA synthetic activity in the cells of the dorsal iris. The increase starts after enhancement of RNA and protein synthesis. DNA synthesis stops when the cells begin to synthesize lens antigens[3] and differentiate into primary lens fibers. Activation of DNA synthesis occurs not only in the dorsal iris cells, which later participate in lens formation, but also in the ventral iris cells, which do not. It is known that in RNA and protein synthesis a similar activation of the ventral iris occurs.[1,2]

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

[2]T. Yamada and C. Takata, *Develop. Biol.* **8**, 358 (1963).

[3]C. Takata, J. F. Albright, and T. Yamada, "Lens Antigens in a Lens-Regenerating System Studied by the Immunofluorescent Technique," *Developmental Biology* (in press).

4.7 STUDY OF TRANSFILTER INDUCTION IN AMPHIBIAN EMBRYOS WITH EXPLANTATION AND AUTORADIOGRAPHY

S. K. Brahma Tuneo Yamada

Introduction. – Regarding the mechanism underlying the process of embryonic induction, there exists two opposing hypotheses: one considers cell contact between the organizer and the ectoderm as indispensable for induction, and the other assumes diffusion of the inducing agents from the organizer to the reacting tissue without any cell contact. Failure of the inductive process to operate through vitelline membrane, cellophane membrane, and also through highly porous membranes

has been cited as evidence in support of the contact hypothesis, while induction by diffusion through agar, Millipore filter, and with soluble fractions prepared from different inductors supports the second hypothesis.[1] In an in vitro system of the amphibian embryonic tissue, it has been reported that primary induction could be obtained through Millipore filter without an actual cell contact.[2] The present experiments were conducted (1) to reinvestigate the problem by employing the same type of filter through which primary induction has been reported[2] and (2) to obtain information on the transfer of compounds related to RNA, whose possible role in the inductive mechanism was suggested by earlier workers.

Materials and Methods. – Early gastrulae of *Triturus pyrrhogaster* were used as the donors of the explants in the following series of experiments. The isolated ectoderm and dorsal mesoderm (organizer) were fused together with a piece of Millipore filter (0.8 μ pore, 25 ± 5 μ thickness) inserted between them. In one series, organizers were incubated in H^3-uridine (50 μc/ml) for 3 hr before making the explants, whereas in the other series, nonlabeled organizers were used. The ectoderm was not labeled in all series. In the control series, two ectodermal pieces were sandwiched with the filter. All these explants were cultured at 17°C for three to four days in Holtfreter solution and then fixed in acetic acid–alcohol. Tissues were studied either by routine histological sections or by autoradiographic sections. Some sections were incubated with RNAse before autoradiograms were made.

Results and Discussion. – In the control series the ectoderm formed only aggregates of epidermal cells and did not show any sign of neural differentiation. In all series in which the ectoderm was combined with the dorsal mesoderm, neural differentiation was observed in the area of the ectoderm which was in direct contact with the mesoderm. However, the part of the ectoderm which was faced to the mesoderm through the filter showed only epidermal differentiation. In conclusion, no transfilter induction was observed.

The autoradiographic study summarized in Table 4.7.1 shows that the average grain counts per unit area are higher in the nucleus than the cytoplasm in all series.

Ribonuclease treatment of the sections comparable to the ones used in Table 4.7.1 removed more than 90% of grains over the cytoplasm of the ectoderm and mesoderm, 30% of grains over the nucleus of the mesoderm, and 82% of grains over the nucleus of the ectoderm.

Table 4.7.1. Grain Counts per Unit Area in Mesodermal and Ectodermal Cells in the Transfilter Experiment

	Nucleus (n)	Cytoplasm (c)	c/c + n
Dorsal mesoderm	51.54 ± 1.87	5.96 ± 2.03	0.103
Neuralized mesoderm	37.86 ± 11.48	4.23 ± 1.02	0.100
Shielded mesoderm	20.73 ± 9.45	3.1 ± 1.26	0.130

The neuralized ectodermal cells which were in direct contact with the labeled mesodermal cells indicated a higher nuclear grain count than the nonneuralized ectoderm which was faced to the mesoderm through the filter. The ratio between nuclear grain counts and cytoplasmic grain counts does not show significant difference among cells of the mesoderm or the neuralized or nonneuralized ectoderm.

The data show that under the condition which allows neural induction, the transfer of labeled substances from the organizer into the ectoderm occurs more intensely than in the presence of filter, which disturbs induction. The data do not support the hypothesis of a mass transfer of cytoplasmic granules[3] containing RNA from the organizer into the ectoderm as a mechanism of organizer action. Since distribution of grains over the neuralized ectoderm cells in the present experiment is not distinguishable from that over the ectoderm cells exposed to H^3-uridine solution, it appears rather likely that transfer of radioactivity from the organizer cells to the ectoderm cells occurred mainly in small molecules which later incorporated into nucleic acids synthesized in the ectodermal nucleus.

[1] L. Saxen and S. Toivonen, *Primary Embryonic Induction*, Logos Press, London, 1962.

[2] L. Saxen, *Develop. Biol.* **3**, 140 (1961).

[3] J. Brachet, *Experientia* **6**, 56 (1950).

4.8 NEURAL INDUCTION AND NUCLEAR RNA SYNTHESIS IN THE ECTODERM OF THE AMPHIBIAN EMBRYO

S. K. Brahma Tuneo Yamada

Introduction. – As an approach to the mechanism of primary embryonic induction, RNA synthesis occurring in the ectoderm which is induced to differentiate into neural tissue under the influence of the normal organizer (dorsal mesoderm) was studied with autoradiography.

Materials and Methods. – The prospective ectoderm was isolated from the early gastrula of *Triturus pyrrhogaster* and separated into halves. One of them was fused to a piece of the dorsal mesoderm isolated from the same embryo (experimental explant). The other half of the ectoderm was used as a control without being combined with the mesoderm. After 1 hr (series *A*) or 48 hr (series *B*) of culture in Holtfreter solution at 17°C, both explants were transferred to H^3-uridine solution (50 μc/ml) and kept for 3 hr. To ensure better penetration of the isotopes, the explants were slit open with sharp tungsten needles before the treatment. Then the explants were fixed, and autoradiographs were prepared. Some slides were treated with RNAse before they were covered with liquid emulsion.

Results and Discussion. – In all explants radioactivity was localized only in the nuclei. Grain counts per unit nuclear area were made in the experimental and control ectoderm of both series and are indicated in Table 4.8.1.

Table 4.8.1. Average Grain Count per Unit Nuclear Area in the Ectodermal Cells

Experimental	Control
Series *A*	
8.04 ± 0.36[a]	5.30 ± 0.77
5.54 ± 0.66[a]	4.74 ± 0.68
12.68 ± 0.87[a]	10.32 ± 0.60
11.74 ± 0.47	10.60 ± 0.45
17.02 ± 0.73[a]	14.24 ± 0.67
11.42 ± 0.66	10.84 ± 0.41
12.38 ± 0.66	14.54 ± 0.49[a]
12.18 ± 0.67	13.38 ± 0.55
Series *B*	
12.62 ± 0.60[a]	8.12 ± 0.44
12.00 ± 0.79[a]	7.18 ± 0.47
13.64 ± 1.07[a]	5.88 ± 0.38
19.32 ± 1.36[a]	10.04 ± 0.71
17.98 ± 1.11[a]	8.32 ± 0.41
19.04 ± 0.99[a]	11.30 ± 0.58
23.02 ± 1.13[a]	7.16 ± 0.41
11.06 ± 0.50	10.66 ± 0.77
21.76 ± 1.49[a]	13.96 ± 0.94

[a]This figure is significantly higher than its counterpart at 95% confidence limits.

In series *A* the count is significantly higher in the experimental than in the control ectoderm in some pairs, but not in others. The RNAse treatment removed 20 to 30% of grains and tended to equilibrate the experimental and control grain counts adjusted for the activity of RNA. On the other hand, in series *B* the count was significantly higher in the experimental than in the control in all pairs of explants except one. Furthermore, 95% of grains present were found removable with RNAse treatment in this series. Thus, data of series *B* as a whole suggest a higher RNA synthetic activity per unit nuclear area in the experimental than in the control ectoderm, while the data of series *A* are not decisive in this respect.

In a special series of experiments, the differentiation capacity of explants in the above autoradiographic slides was tested by culturing them in Holtfreter solution for 72 hr. In 80% of explants containing the ectoderm fused to the organizer, neuralization of the ectodermal component occurred. By contrast, all the ectodermal explants without the added organizer differentiated epidermal cells alone. According to Gillette,[1] at the developmental stages concerned no difference exists in the cell size in the prospective neural ectoderm and prospective epidermal ectoderm. Further studies show that no significant difference is present in the nuclear size between both types of ectoderm.

Thus, the data so far obtained suggest that when the ectoderm is induced to differentiate neural tissue under the influence of the dorsal mesoderm, an enhancement of RNA synthesis in the nucleus precedes neural differentiation.

[1]R. Gillette, *J. Exptl. Zool.* **96**, 201 (1944).

4.9 LAMPBRUSH CHROMOSOMES – SOME ASPECTS OF FINE STRUCTURE

O. L. Miller, Jr.

Introduction. – The lampbrush chromosomes of amphibian oocytes offer a unique opportunity for studying the structural relationship between genetically active DNA and its transcriptive products. These chromosomes are readily isolated by manual techniques and can be briefly described as follows. They are long (0.3–1.0 mm) diplotene bivalents with homologous main axes joined at one to several points by chiasmata. The main axes consist of numerous Feulgen-positive chromomeres, from which pairs of lateral loops extend. Such loops range up to 200 μ in length and consist of a thin axial fiber surrounded by an RNA-protein matrix. Matrix morphology is constant and similar for loops of homozygous chromomeres but may differ from locus to locus. Enzyme-breakage kinetics suggest that the linear continuity of loop axes is maintained laterally by a single, double-helix DNA molecule, while the interchromomeric fiber of the main axes contains two DNA molecules. Labeling with radioactive precursors shows that continual synthesis of both RNA and protein occurs on the lateral loops.

This is a report of preliminary attempts to study the structural relations between loop axes and matrices on lampbrush chromosomes from the spotted newt, *Triturus viridescens*.

Method. – The nucleus of an immature oocyte is isolated with jewelers' forceps into saline and washed free of yolk with a braking pipet. At this point, the nucleus may be treated with various agents, changes in pH or molarity, etc., which affect the structure of the chromosomes when still in the nucleus. Next, the nucleus is pipetted into a small drop of saline or a 10% sucrose solution. If sucrose is used, the nucleus usually will rise until the nuclear envelope attaches to the surface film; with saline, the drop is pulled down until the surface film contacts the nucleus. In both cases, surface tension forces burst the envelope and pull it, as well as a portion of the chromosomes, into the surface film. The chromosomes in the surface are picked up on a carbon-coated electron microscope grid by touching a grid to the surface of the drop. The preparation is rinsed with distilled water, stained with saturated uranyl acetate solution, dehydrated with EtOH, and examined in the electron microscope.

Results and Discussion. – Chromosome breakage occurs in the surface film, preventing identification of specific chromosomes. However, long portions of main axes with loops can be obtained on grids. In addition to breakage, the surface film action removes loop matrix, providing a gradient ranging from loops retaining essentially all of their matrix to loops nearly devoid of matrix. Intact matrices are electron dense and highly irregular in outline, varying from an average diameter of 0.2 μ near the thin chromomeric insertion point to 0.6 to 1.0 μ over the rest of the loop. Examination of partially denuded loops shows that the matrix material is composed of tightly packed subunits, also irregular in outline, with an average diameter of 0.5 μ. In places where matrix has been completely removed from loops, an axial fiber 40–100 A in diameter is exposed. In some instances short lengths of this fiber are too thin and/or electron transparent to be well resolved. The fiber can be broken by DNAse but not RNAse or proteases (phase microscopy observations). Small, dense granules of varying diameter generally are found in irregular association with the axial fibers. In many instances, however, groups of 5 to 20 granules (500 A diam) are attached to the axis with a periodicity of about 1300 A.

At the outer end of loops from which much of the matrix has been removed, one or more matrix subunits usually are found partially to completely uncoiled and ranging up to 7 μ in length. In this extended form the subunit appears to consist of a very thin fiber irregularly covered along its length with small, electron-dense clumps and particles. The spacing of matrix fibers approaches that of the regularly spaced axial granules, suggesting that the granules may be sites of matrix attachment.

Since loop matrices contain RNA which is synthesized in situ, the long fibrous component of the matrix subunits possibly is transcriptive RNA. In this case the longer subunits (7 μ) would have a triplet-coding potential for 3500 amino acid residues (i.e., a polypeptide of about 0.5×10^6 mol. wt). Furthermore, if the spaced axial granules in this material are sites of RNA attachment to DNA, a length of DNA necessary to transcribe an RNA molecule capable of templating a large protein molecule has approximately 50 RNA molecules of corresponding length attached to it.

4.10 EXPANDABLE LOOP TECHNIQUE FOR ELECTRON MICROSCOPY–AUTORADIOGRAPHY EMULSION FILMS

O. L. Miller, Jr. Bessie G. Lukes

Introduction. – One of the technical problems inherent in high-resolution electron microscopy–autoradiography (EM-ARG) is the consistent application of thin emulsion films composed of homogeneous monolayers of closely packed silver halide crystals. As stressed by Caro,[1] the application of liquid emulsion results in an uneven distribution of crystals owing to capillarity between the grid wires and the emulsion. If the emulsion film is allowed to gel immediately preceding application, however, the crystals remain in a relatively homogenous layer over the entire grid surface.

Since films made with concentrated liquid emulsion are too thick for EM-ARG, attempts to obtain monolayers usually have involved diluting emulsion with two to several parts of distilled water. The innovation in technique described in this report is the expansion of a small amount of undiluted emulsion into a thin film which is allowed to gel before applying to EM grids.

Technique. – An expandable wire loop is produced by attaching one end of a 2- to $2\frac{1}{2}$-ft length of nickel-chrome alloy wire (size 22) to the end of a hard-glass, fine-bore pipet with masking tape. (Alternatively, a disposable plastic syringe, with the plastic needle cover retained but needle and plunger removed, may be used.) The free end of the wire is inserted into the bore and pulled through the pipet until a minimum diameter loop is attained.

Undiluted Ilford L4 nuclear track emulsion is melted in a 40–45°C water bath and then cooled to 30°C. The small loop is immersed in the emulsion and gently withdrawn with a film of concentrated emulsion. The loop is carefully but rapidly expanded under an amber safelight (Wratten series OC) until the film displays dull first-order interference colors. After 5–10 sec, the interference colors suddenly brighten, indicating that the film has gelled. The area of the film showing suitable homogeneity and thinness is touched to the surface of EM grids which have been mounted (with sections up) on a microscope slide. The stickiness of the gelled film causes even adherence to the surface of the slide and grids. The slides are stored in lighttight boxes for exposure. A variety of photographic processing and staining procedures are available for preparing the autoradiographs for microscopy.

Discussion. – Electron microscopy examination of dried but undeveloped emulsion films produced by this technique shows homogeneous monolayers of crystals. Thicker films of known dimension can be obtained by using higher-order interference colors. The utility of the technique has been tested in several EM-ARG studies[2] (see also Sects. 4.4 and 10.5).

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[1] L. G. Caro and R. P. van Tubergen, *J. Cell Biol.* **15**, 173 (1962).

[2] A. R. Stevens, *J. Cell Biol.* **19**, 3A (1963).

4.11 HISTONES IN THE REGENERATING LENS OF *TRITURUS VIRIDESCENS*

Ann Andrew Vivian Gabie

Introduction. – Since histones are thought to function as gene suppressors, it has been postulated that they play an important role in differentiation. With this view in mind, a histochemical analysis of histones in a differentiating system, the regenerating lens, was undertaken.

Materials and Methods. – Eyes of adult *Triturus viridescens* were fixed at intervals between 4 and 25 days after lens removal. Slides were prepared for the histochemical identification of lysine- and arginine-rich histones as well as protamines, according to the methods used by Alfert and Geschwind[1] and Bloch and Hew.[2] Sections of normal eyes were prepared in the same way.

Results. – Staining of lysine-rich histones shows a slight, but definite, decrease in intensity in the nuclei of the dorsal iris margin, reaching the lowest level during stages II and III (6–12 days). (Staging is according to Stone and Steinitz.[3]) This is followed by an increase which may start at stage IV (12 days) but is certainly well on the way in stage VI (15 days). Thereafter, whereas the nuclei of the primary lens fibers remain lightly stained, staining of the nuclei of the lens epithelium continues to darken. Nuclei of epithelial cells transforming into secondary lens fibers (stage IX, 19 days onward) become lightly stained.

Even by stage XI (25 days) staining of the nuclei of the lens epithelium has not attained the intensity found in the nuclei in the presumptive lens region at stage I (4 days) nor in the nuclei of the normal lens epithelium.

Granules of stained material are more easily seen in the nuclei of the regenerating lens at the time when the overall nuclear staining is least intense. It is possible that they may be masked at other times by more diffusely staining material. If this is so, these observations may signify differences in the composition and distribution of histones.

There is no evidence of any arginine-rich histones or protamines at any of the above stages.

The staining of lysine-rich histones in the nuclei of the ventral border of the iris shows a pattern of decrease and subsequent increase somewhat similar to the regenerating region.

Observations on the occurrence of lysine- and arginine-rich histones and protamines were also made on the remainder of the iris, the cornea, and the retina in operated eyes, and comparisons were drawn with normal eyes. No significant differences were apparent.

Discussion. – A decrease in intensity of staining of lysine-rich histones may be due to a dilution effect if nuclear size increases simultaneously. The iris nuclei are known to increase in size by a factor of 1.8 during the first 16 days of lens regeneration.[4] That this is a continuous increase is suggested by the continuously rising nucleocytoplasmic ratio described during this period.[5] Since an initial decrease in lysine-rich histone staining is followed by an increase during this same period, a dilution effect alone would not explain the histochemical results obtained. Therefore, there may be a real decrease in lysine-rich histone content during the early stages of lens regeneration.

Huang and Bonner[6] have shown that more RNA is synthesized by DNA free from histones than by DNA associated with histones. In view of their results, the observed decrease and subsequent increase in the amount of lysine-rich histones during early stages of lens regeneration may be related to changes in RNA synthesis. An enhancement of RNA synthesis has been reported[4] during the first 16 days of lens regeneration, but no details of fluctuations are available. Likewise the decrease in lysine-rich histone level in the ventral iris margin may be related to the increase in nuclear RNA synthesis observed[4] in the ventral iris margin and perhaps to the recorded increase in protein synthesis.[7]

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[1] M. Alfert and I. I. Geschwind, *Proc. Natl. Acad. Sci. U.S.* **39**, 991 (1953).

[2] D. P. Bloch and H. Y. C. Hew, *J. Biophys. Biochem. Cytol.* **8**, 69 (1960).

[3] L. S. Stone and H. Steinitz, *J. Exptl. Zool.* **124**, 435 (1953).

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

[5] S. Karasaki, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 62.

[6] R. C. Huang and J. Bonner, *Proc. Natl. Acad. Sci. U.S.* **48**, 1216 (1962).

[7] T. Yamada and C. Takata, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 64.

5. Mammalian Genetics

Section Chief – W. L. Russell

Genetic Effects of Radiation in Mice (5.1–5.2)		Mammalian Cytogenetics (5.3–5.4)	
U. H. Ehling	Jean W. Bangham	Liane B. Russell	Clyde S. Montgomery
Elizabeth M. Kelly	Patricia A. Burd	B. M. Cattanach[b]	
Louis Wickham	Georgia M. Guinn		
J. R. Inman	Patricia R. Hunsicker	**Effects of Radiation on Mammalian Gametogenesis (5.5)**	
Josephine S. Gower[a]	Martha M. Larsen	E. F. Oakberg	Evelyn Clark
	Savanna C. Maddux		M. Jo Henderson
	Elizabeth L. Phipps		
	Jackie N. Poole	**Cooperative NIH-AEC Chemical Co-Carcinogenesis Program (5.4)**	
	Mary H. Steele		
	Kathren F. Stelzner		
	H. M. Thompson, Jr.		
	Carolyn M. Vaughan		
	Sandra J. Weaver		
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5.1 RELATIVE BIOLOGICAL EFFECTIVENESS OF NEUTRON TO X-RAY INDUCTION OF GENE MUTATIONS IN MOUSE POSTSPERMATOGONIAL STAGES

W. L. Russell Elizabeth M. Kelly

For several years we have been receiving urgent requests from national and international bodies to obtain data on the induction of gene mutations by neutrons in mice. Estimates of genetic hazards of neutron irradiation in man had to be based on theory or on extensive extrapolation from limited information. The only neutron data on gene mutation in mammals was that obtained by us from the small numbers of animals we had been able to place in a cyclotron beam or to enclose in lead chambers exposed to a nuclear detonation. Recently, however, a suitable facility finally became available to us. This is the ORNL Health Physics Research Reactor (HPRR),[1] which, through the excellent cooperation of the Health Physics Division, we were able to use for mouse mutation experiments as soon as construction and testing were completed. The HPRR is a small fast reactor similar to the Godiva II reactor at Los Alamos[2] and the Sandia Pulsed Reactor Facility.[3] The reactor core, which is suspended beneath the reactor framework, can be positioned remotely in the center of a large rack of mouse cages. The fast neutron to gamma radiation tissue dose ratio is approximately 10; the thermal neutron dose to mice is less than 1% of that due to fast neutrons for the irradiation geometry used, and the neutron spectrum approximates a fission spectrum. One great advantage of this reactor is that it can be used to test a wide range of dose rates. Ever since our discovery of a dose-rate effect of x and

gamma radiation on mutation, it has been of vital importance, both from the hazard point of view and for an understanding of the fundamental nature of the dose-rate effect, to know whether or not a similar effect occurs with neutron irradiation.

Using our standard specific locus method, we have already obtained nearly 100 mutations in a series of neutron experiments involving various doses and dose rates in both sexes. A report on the preliminary results from all these experiments is in preparation. The information obtained from the portion of the investigation that deals with mutations induced in postspermatogonial stages is complete, apart from final testing of the mutations, and is reported here. Mutations induced in postspermatogonial stages are observed in the offspring conceived from matings made in the first six weeks following irradiation of the males. The number of females available permitted mating of only one of the dose groups (112–123 rads) immediately after irradiation. This group contained a total of 1148 males irradiated at three different dose rates. It was possible to mate another dose group (63 rads) of 767 males for the latter portion of the initial six-week period. The results are shown in Table 5.1.1. The neutron dosimetry, carried out by members of the Health Physics Division, was based on measurements taken with a Hurst proportional chamber[4] and normalized for each separate exposure to the activation of a sulfur pellet located on the reactor. Initially, the Hurst chamber was calibrated with a 1-curie Pu-Be source using a dose conversion factor of 4.0×10^{-9} rad neutron^{-1} cm^{-2}.[5] Then, the chamber was set up at the experimental position and exposed simultaneously with a normalizing sulfur pellet for 1 hr at a power level of approximately 0.1 w. The resulting neutron dose rate was found to be 3.24 millirads per unit activation of the sulfur pellet. This value was used to calculate first-collision neutron doses for each experimental run based on the sulfur pellet normalizations.

It is apparent in Table 5.1.1 that there is no significant effect of dose rate, and none was expected in view of the absence of any such effect for x and gamma irradiation of the late postspermatogonial stages. For the estimation of relative biological effectiveness (RBE) values the results from the different dose rates are, therefore, pooled for comparison with the data from several of our x-ray experiments shown in Table 5.1.2. Of the various comparisons possible, the one that is most valid, biologically, for the determination of RBE is that between the 600-r x-ray data and the results for the higher neutron dose group. The mutation rate is similar in these two sets of data, and other factors, which need not be gone into in detail in this brief account, were matched so that the array of postspermatogonial stages represented (probably mostly spermatozoa and spermatids) should be similar in the two experiments. Calculating the RBE of neutrons to x rays on this basis, with allowance for the gamma component in the reactor exposures

Table 5.1.1. Specific Locus Mutations Induced by Neutron Irradiation of Postspermatogonial Stages in Mice

Neutron Dose[a] (rads)	Neutron Dose Rate (rads/min)	Number of Offspring	Number of Mutations at 7 Loci
63	0.84	880	1
63	85	766	2
123	0.17	1410	4
115	0.79	1468	3
112	82	1528	5

[a]Total dose would include a gamma component equal to approximately 10% of the neutron component.

Table 5.1.2. Specific Locus Mutations Induced by X Irradiation of Postspermatogonial Stages in Mice[a]

Dose (r)	Number of Offspring	Number of Mutations at 7 Loci
0	531,500	28
300	36,896	51
600	10,665	24
1000	631	4

[a]Dose rate: approximately 90 r/min throughout except for the 600-r data, in which slightly less than one-half of the results came from experiments at 9 r/min.

and with conversion of the x-ray doses to rads, we obtain a value of 5.8. This has rather wide 95% confidence limits of 2.3 and 11.7, but the likelihood that the true value does not deviate much from the point estimate is strengthened by the rest of the data. Thus, comparisons using all the neutron and x-ray results, or other portions, such as the data from the first two weeks of mating (coming, probably exclusively, from irradiated spermatozoa), give RBE's all within the range from 4.5 to 5.5. These figures are also in rough agreement with the RBE for acute irradiation of spermatogonial stages, as will be seen in the report on these now in preparation.

[1]M. I. Lundin, *Health Physics Research Reactor Hazards Summary*, ORNL-3248 (Aug. 24, 1962).

[2]T. F. Wimett *et al.*, "Godiva II – An Unmoderated Pulse-Irradiation Reactor," *Nucl. Sci. Eng.* **8**, 691–708 (1960).

[3]R. D. O'Brien, *Hazards Evaluation of the Sandia Pulsed Reactor Facility* (SPRF), SC-4375A (RR) (February 1961).

[4]E. B. Wagner and G. S. Hurst, *Rev. Sci. Instr.* 29(2) 153 (1958).

[5]T. R. Jones, D. R. Johnson, and J. H. Thorngate, "Neutron Dose Conversion Factors for a Pu-Be and Po-Be Source," submitted to *Health Physics*.

5.2 MUTATION FREQUENCY OF DOMINANT SKELETAL ABNORMALITIES IN MICE

U. H. Ehling

Introduction. – A recent experiment demonstrated the feasibility of measuring part of the overall genetic damage of irradiation by skeletal studies in the F_1 generation.[1] The skeleton was chosen because it is formed over an extended period of development and is, therefore, presumably subject to modification by gene action falling within a wide range of time. The present experiment was designed to estimate the frequency of presumed dominant mutations affecting the skeleton.

Methods. – Male 101-strain mice were irradiated with 600 r (80 r/min). After irradiation, each male was mated to three C3H females. The males were transferred to a fresh set of females 7, 14, and 21 days after irradiation. Nonirradiated control males were mated in the same manner. Litters were sacrificed at the age of 26 to 28 days. Nine hundred sixty-one whole skeletons were examined in alizarin red S preparations.

Results and Conclusion. – The classification used to separate, as far as is possible, the existing natural variability from that caused by newly occurring genetic changes was described elsewhere.[1] It is assumed that the sample size is such that not more than one mutation would be expected for any particular gene locus, and, therefore, that the distinction could best be made by merely dividing the abnormalities according to whether they occurred only once in the whole experiment (class 1) or more than once (class 2). With this classification a statistically significant increase in class 1 abnormalities of the different subgroups is expected to provide the most sensitive indication of mutations. The pooled frequency of class 1 abnormalities of all experiments after exposure of the males with 0 or 600 r is given in Table 5.2.1.

No significant differences in the frequency of class 2 abnormalities and class 1 abnormalities of the single type of the axial skeleton or the unilateral type of the appendicular skeleton between offspring derived from irradiated and nonirradiated males were observed. While it is possible that the number of skeletons examined was not large enough to detect such differences, it appears likely that the majority of these abnormalities are the result of nongenetic factors. The highly significant increase of class 1 abnormalities of the multiple type of the axial skeleton, and the indication of some increase for the bilateral type of the appendicular skeleton, in offspring from irradiated males, suggest that the majority of these anomalies is due to induced dominant mutations. If this assumption is correct, the yield of dominant mutations induced in postspermatogonial stages is 2.9×10^{-5} gamete/r for mutations with skeletal defects detectable by the present method.

[1]U. H. Ehling and M. L. Randolph, *Genetics* **47**, 1543 (1962).

Table 5.2.1. Frequency of Class 1 Abnormalities and of Presumed Dominant Mutations

Dose (rads)	Stage	Number of Offspring	Abnormalities of Axial Skeleton		Abnormalities of Appendicular Skeleton		Number of Presumed Mutations
			Multiple	Single	Bilateral	Unilateral	
0		1131	0	6	0	6	0
600	Postgonial	569	7	5	3	2	10
600	Gonial	157	1	1	0	0	1
P value[a]			0.0005	0.64	0.06	0.33	

[a]Control group vs irradiated groups.

5.3 TIME OF DEATH OF MICE HOMOZYGOUS FOR SMALL DEFICIENCIES

Liane B. Russell Patricia R. Hunsicker

Introduction. – Among specific loci used in mutation rate experiments are the closely linked markers *d* (dilute) and *se* (short ear), recombination = 0.16%, which were included for the detection of deficiencies.[1] Following x or gamma irradiation of spermatogonia, mutations in that region are almost exclusively *d* or *se*, and only rarely *dse*; but irradiation of other cell stages yields a relatively higher ratio of double (*dse*) to single (*d* or *se*) mutants.[2,3] In an earlier publication,[3] we reported detailed studies on 15 of these "double mutants." In the meantime, many more have occurred, and the total number now stands at 37. Of these, 20 have bred consistent with the idea that they represent deficiencies; 6 are cases of what we have called "double nondisjunction" (i.e., they appear to bear two doses of test-stock *dse* chromosome and no wild-type chromosome from the other parent); 3 died before they could be fully tested; and 8 are still in process of being tested. All 20 deficiencies are homozygous lethal before birth. In the case of 9 of them, embryological work is in progress to determine time of death. The present report will present partial data on 7 of these as well as on a single-mutant *se* lethal which is being studied for comparison.

Methods. – For each of the *dse* stocks investigated, embryonic mortality is being compared in a "control" and an "experimental" cross in which the mothers are kept constant, being deficiency heteroyzgotes. The crosses are as follows:

for "control,"

$$+\ se/'dse'\ ♀ \times d+/dse\ ♂;$$

for "experimental,"

$$+\ se/'dse'\ ♀ \times d+/'dse'\ ♂ \text{ (of same stock as ♀)},$$

where single quotation marks around *dse* represent the deficiencies. Thus, the embryos, in each case, are of four possible genotypes with respect to the *d-se* region, one of these, in the case of the experimental cross, being the deficiency homozygotes. Furthermore, in each cross, two classes of embryos are deficiency heterozygotes.

Matings are timed by the observation of vaginal plugs. The mothers are sacrificed on day 15½ following insemination, at which time the uterine contents are examined and corpora lutea counted. Results to date are shown in Table 5.3.1.

Discussion of Results. – It may be noted that, even in control matings, embryonic loss is considerably higher than that in females of a good stock. Thus, in (101 × C3H)F_1 females, for instance, the percentage of corpora lutea not represented by living embryos is only about 15%,[4] while in the stocks used here it ranges from 21

Table 5.3.1. Intrauterine Findings and Corpora Lutea Counts in Matings of Mice Carrying *dse* Deletions

Mutation	Stock	Type of Mating	Number of						Ratios	
			Uteri	Corpora Lutea	Living Embryos	Late Deaths[a]	Early Deaths[b]	Preimplantation Losses[c]	Preimplantation Loss/Corpora Lutea	Postimplantation Loss/Corpora Lutea
'*dse*'	209G	C	13	99	59	2	11	27	0.27	0.13
		Ex	18	146	76	1	5	64	0.44	0.04
		Ex-C							0.17	−0.09
	17Zb	C	15	125.5[d]	89	0	13	23.5	0.18	0.10
		Ex	15	144	84	1	8	51	0.35	0.06
		Ex-C							0.17	−0.04
	3SAS	C	14	111	79	1	15	16	0.14	0.14
		Ex	15	118.5	69	0	21	28.5	0.24	0.17
		Ex-C							0.10	0.03
	37FBFo	C	8	63.5	33	0	2	28.5	0.45	0.03
		Ex	12	109.5	48	0	3	58.5	0.53	0.03
		Ex-C							0.08	0.00
	130G	C	6	55.5	44	1	3	7.5	0.13	0.07
		Ex	5	39	29	0	2	8	0.20	0.05
		Ex-C							0.07	−0.02
	43CoS	C	18	154.5	110	1	8	35.5	0.23	0.06
		Ex	15	126.5	80	0	11	35.5	0.28	0.09
		Ex-C							0.05	0.03
	4DTD	C	13	105	66	0	13	26.0	0.25	0.12
		Ex	15	108.5	63	2	27	16.5	0.15	0.27
		Ex-C							−0.10	0.14
'*se*'	207K	C	14	117.5	84	2	12	19.5	0.17	0.12
		Ex	16	132.5	69	0	28	35.5	0.27	0.21
		Ex-C							0.10	0.09

[a]Death later than day $10\frac{1}{2}$ postinsemination.
[b]Death between implantation and day $10\frac{1}{2}$.
[c]Difference between corpora lutea and all implants, living and dead.
[d]Doubtful corpora lutea are scored as 0.5.

to 48%. It is possible that the intrauterine environment is somewhat adversely affected in females heterozygous for '*dse*' deletions. It had already been noted earlier that at least some of these deletions have slight dominant effects on size, particularly in the + *se*/'*dse*' compounds.[3]

The theoretical expectation of 25% excess death in experimental matings was not reached in any of the stocks (see Ex-C for the ratios in Table 5.3.1). This is, however, the usual experience with lethal genes in the mouse, since early death of homozygotes leads to a decrease in competition for survival among embryos of the other classes. There is an additional factor to be considered in the present experiment, namely, the probable decrease in transmission of '*dse*' gametes that may occur at least in some stocks.[3] Although none of the stocks chosen for the embryological work is in the very low transmission class, some of them (209G, 3SAS, 130G) have given evidence of slightly reduced transmission.

As to time of death, it may be noted that "late" death is very rare, both in experimental and in control matings. Early postimplantation death, which occurs more frequently, shows no consistent excess in experimental matings, except in the case of the '*se*' mutation 207K and possibly in one of the deficiencies, namely, 4DTD. Preimplantation loss, on the other hand, is consistently in excess in experimental matings (except in 4DTD). On the basis of these incomplete results, it may be tentatively concluded that embryonic death in the case of most *dse* deficiencies occurs *before* implantation, while an *se*-lethal, included for comparison, kills around the time of implantation (losses about equally divided between preimplantation and early postimplantation).

It may be noted that none of the *dse* deficiencies is cytologically visible in mitotic metaphase and may, therefore, be presumed to involve short or medium lengths of chromosomal material. Determinations of genetic length have been started.

Conclusions. – The evidence so far would seem to indicate that genetic damage from what are probably relatively small homozygous deficiencies can be greater than that from heterozygous losses of entire chromosomes (probably the bulk of radiation-induced "dominant lethals") which cause death both before and after implantation.

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

[2]W. L. Russell and L. B. Russell, *Radiation Res., suppl. 1*, 296 (1959).

[3]L. B. Russell and W. L. Russell, *J. Cellular Comp. Physiol., suppl. 1*, **56**, 169 (1960).

[4]W. L. Russell, L. B. Russell, and A. W. Kimball, *Am. Naturalist* **88**, 260 (1954).

5.4 CHEMICAL MUTATION STUDIES IN MICE[1]

B. M. Cattanach

Introduction. – It has been shown in the mouse that treatment of either spermatozoa or spermatids with triethylenemelamine (TEM) will induce both dominant lethal mutations and translocations.[2,3] The frequency of dominant lethals following treatment of spermatocytes, however, is extremely low. This can in part be explained by the fact that spermatocytes before differentiating to give spermatozoa must undergo meiosis – a process that would be expected to select out most chromosomal changes that cause dominant lethality. Translocations, on the other hand, are capable of surviving meiosis, for they may be transmitted from one generation to another. This type of chromosome change, when induced in spermatocytes, should therefore be recoverable.

The present report describes the results of tests for translocations induced in mouse spermatocytes by TEM.

Material. – According to Oakberg's timing of spermatogenesis in the mouse,[4] spermatozoa present in the ejaculate 22 to 42 days after treatment should be derived from cells that were spermatocytes at the time of treatment. F_1 sons, conceived between 22 and 42 days of treatment of the fathers, were therefore collected to test for translocations. The source of the F_1 animals was an experiment to test for TEM-induced spermatogonial gene mutations.[5] The treated males were 101 × C3H hybrids and had received a single dose of 1.0 mg of TEM per kg of body weight.

Results. – One hundred seventeen F_1 sons were collected and tested for translocation semisterility. Of these, seven were selected as being semisterile, and a further three were completely

sterile. The meiotic metaphase-I figures of all 10 animals were examined to confirm the presence of any translocation. Mitotic metaphases of several of the animals were also studied.

Male No. 1: Semisterile. Conceived on day 25. Meiotic metaphase I – 16 bivalents plus a ring of 8 were seen in 80% of the cells. Interpretation: four chromosomes have been broken, and the exchanges were such that chromosome A rejoined with B, B with C, C with D, and D with A. This is equivalent to two translocations and is unique only in the distribution of the broken ends.

Males Nos. 3, 7, and 8: Semisterile. Conceived on days 23, 24, and 24 respectively. Meiotic metaphase I – 18 bivalents plus an association of 4 were consistently seen. Interpretation: single reciprocal translocations.

Males Nos. 16, 30, and 63: Semisterile. Conceived on days 26, 31, and 31 respectively. Meiotic metaphase I – 20 bivalents always seen. Interpretation: chromosomally normal.

Male No. 6: Sterile. Conceived on day 23. Meiotic metaphase I – 18 bivalents, an association of 3, and a univalent were seen in the majority of the cells. Mitotic metaphase – one unusually long and one unusually small chromosome could be identified. Interpretation: a single reciprocal translocation in which the exchange is very asymmetrical – a long piece of chromosome being exchanged for a short piece.

Male No. 9: Sterile. Conceived on day 24. Meiotic metaphase I – 16 bivalents, an association of 4, an association of 3, plus a univalent were regularly observed. Mitotic metaphase – 3 rearranged chromosomes could be identified. One was twice as long as any other chromosome, one was extremely small, and one was slightly larger than any other normal chromosome. Interpretation: two separate reciprocal translocations in one of which the exchange is very asymmetrical as in male No. 6.

Male No. 18: Sterile. Conceived on day 27. Meiotic metaphase I – 20 bivalents plus a univalent were regularly seen. Mitotic metaphase – 41 elements were always counted. Interpretation: trisomy or partial trisomy. The extra chromosome appears to be in the small size range, this probably accounting for the absence of trivalent formation during meiosis.

Discussion. – Out of 117 animals tested, 6 were found that together possessed a total of 8 translocations. The frequency of recovered translocations is therefore 6.8%.

It must be noted that all the animals possessing rearrangements were conceived early in the 22 to 42 day period, that is, between the 22d and 25th days. This could be interpreted as meaning that translocations may only be induced, or may only be recovered, from late spermatocytes. A more likely explantation, however, is that the rearrangements were induced in spermatids rather than in spermatocytes. The data would then constitute evidence that spermatozoa which were spermatids at the time of treatment may be present in the ejaculate up to 25 days after treatment. Although there is no previous evidence for a mixing of spermatozoa derived from different spermatogenic stages, this possibility is made more likely by the fact that the TEM treatment stopped spermatogonial production.[6] With the lack of any pressure from behind, spermatogenic cells may be delayed in their passage through the seminiferous tubules.[7]

The data cannot satisfactorily be taken as evidence for the induction of translocations by TEM in spermatocytes, but the cytological analysis of the rearrangements was extremely interesting. The multiple translocation giving rise to a ring of 8 in male No. 1 does not require any new mechanism for its production, but the probability of this event occurring must be extremely small. Both the single translocation in male No. 6 and the two translocations in male No. 9 are interesting in that they show clearly identifiable marker chromosomes in mitotic as well as in meiotic cells. It is unfortunate that both these animals were completely sterile, for, with their chromosome markers, they would have had a variety of uses.

The most fascinating observation was that one male (No. 18) possessed 41 chromosomes. This must be the first case of viable autosomal trisomy, or partial trisomy, in the mouse. It cannot be proved that meiotic nondisjunction of a chromosome was the consequence of the TEM treatment, but it should be noted that this animal was conceived at a time when, almost certainly, the spermatocyte was the cell stage being tested.

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[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]B. M. Cattanach and R. G. Edwards, *Proc. Roy. Soc. Edinburgh, B*, **67**, 54 (1958).

[3]B. M. Cattanach, *Z. Vererbungslehre* **90**, 1 (1959).

[4]E. F. Oakberg, *Am. J. Anat.* **99**, 507 (1956).

[5]B. M. Cattanach, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 56.

[6]B. M. Cattanach, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 78.

[7]E. F. Oakberg and R. L. DiMinno, *Int. J. Rad. Biol.* 2(2), 196 (1960).

5.5 COMPARISON OF SPERMATOGONIAL POPULATIONS SURVIVING SINGLE AND FRACTIONATED X-RAY DOSES

E. F. Oakberg Evelyn Clark
M. Jo Henderson

Cell synchronization by the first dose of radiation has been postulated as a possible explanation of the high mutation rate obtained when 1000 r is given as two 500-r fractions 24 hr apart.[1] In the previous progress report,[2] it was shown that most spermatogonia are in G_1 of interphase 24 hr after exposure to 500 r. Postirradiation DNA synthesis and differential survival of cells in S and G_2 have been studied after single exposures of 500 and 1000 r, and of 1000 r given in two 500-r fractions 24 hr apart in order to further evaluate the possible effect of disturbances in the spermatogonial population on mutation frequency.

Male mice (101 × C3H), approximately 12 weeks old, were used. Tritiated thymidine was injected either 30 min before irradiation, in order to score survival of cells irradiated during DNA synthesis, or 6 hr before irradiation, in order to label cells irradiated during the G_2 phase. DNA synthesis after irradiation was studied by injection of H^3-thymidine 30 min before the mice were killed. Autoradiographs of 5-μ paraffin sections were prepared and scored. Any cell with three or more silver grains above the nucleus was classified as labeled.

No differences in survival between *S* and G_2 were observed at one day (Table 5.5.1); and in all exposures, labeled cells have been lost more rapidly than unlabeled, since the values are much below control. At five days, however, the response is dependent on dose, with higher survival of G_2 than S after the single exposures, and equal survival of S and G_2 after the fractionated dose. The proportion of the surviving population still labeled at five days was higher for 1000 than for 500 r, and suggests a long mitotic arrest. More data are needed before these results can be considered established, but even if substantiated, the differences are too small to explain the mutation frequencies observed by Russell[1] on the basis of differential cell survival alone.

Table 5.5.1. Survival of Type A Spermatogonia in Mice Injected with H^3-Thymidine (I.P.) and Irradiated in in S and G_2 Phases

Dose (r)	Time Killed (days after irradiation)	Survival (%)	
		S Phase[a]	G_2 Phase[b]
0 (control)		21.4[c]	25.8[c]
500	1	6.3	9.4
500	5	3.2	8.4
500 + 500	5	5.7	5.8
1000	1	7.4	5.1
1000	5	9.1	19.3

[a]Injected 30 min before irradiation.
[b]Injected 6 hr before irradiation.
[c]Percent labeled cells.

Table 5.5.2. Percentage of Type A Spermatogonia Labeled by Postirradiation Injection of H^3-Thymidine

Time After Irradiation (days)	Percent Labeled		
	500 r	500 r + 500 r	1000 r
0 (control)	21.4	21.4	21.4
1	2.1	7.3	1.0
2	8.2	11.8	3.7
3	20.1	16.3	9.5
4	25.8	31.6	15.8
5	40.9	19.9	28.3
6	35.3	30.7	23.5
7	34.7	37.8	29.4
10	24.2	28.2	35.2

DNA synthesis was very low one and two days after all doses (Table 5.5.2), with a subsequent rise above control frequency of labeling. Neither the single nor the fractionated 1000-r exposure was followed long enough for return to control values. These data are difficult to interpret owing to possible failure of lethally damaged cells to synthesize DNA (especially at early postirradiation times) and by subsequent degeneration of cells that do label. The above-normal labeling at four to seven days for groups given 500 and 500 + 500 r and seven to ten days for 1000-r groups is in support of our earlier conclusion that most surviving cells are in G_1. The possibility of a prolongation of S, and even of the entire cell cycle, has not been excluded, however. Differences between the three groups in the degree of perturbation of cellular metabolism as revealed by H^3-thymidine incorporation are suggested by the data of Table 5.5.2. More information is required, however, in order to assess the possible contribution of these disturbances in metabolism to mutation frequency.

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6. Radiation Microbiology

Alexander Hollaender	
Mutagen Mechanisms – Bacterial Metabolism (6.1–6.2) G. E. Stapleton[a] Annie S. Angel Roberto Masironi[b]	**Bacterial Genetics (6.3–6.6)** H. I. Adler[a] L. J. Charamella Roy Curtiss III Alice A. Hardigree D. E. Duggan[c] Claire M. Berg[d]
[a]Dual assignment. [b]Visiting investigator from Italian Atomic Energy Committee. [c]Visiting Investigator from Canada on USPHS Fellowship. [d]Loanee.	**Cooperative NIH-AEC Chemical Co-Carcinogenesis Program (6.4)**

6.1 DNA CONTENT AND RADIORESISTANCE IN *ESCHERICHIA COLI*

G. E. Stapleton Annie S. Angel

Several reports suggest that unbalanced growth resulting in increased DNA content per cell leads to enhanced resistance to inactivation of *Escherichia coli* by ultraviolet[1,2] and also by x rays.[3,4] The imbalance has usually been accomplished in the 15_{TAU-} strain by supplying thymine to growing cultures of this polyauxotrophic strain and withholding its other two requirements, arginine and uracil. Under these conditions, cells of this strain are reported to increase their DNA content by about 40% in 60 to 90 min at 37°C. Since little or no RNA or protein synthesis occurs during the same period, the ratio of DNA to RNA or protein increases to about 1.4. Cells from such unbalanced growth are more resistant to radiations than are growing cells with balanced synthesis. Their survival curves reflect a change in shoulder (extrapolation number) rather than in slope.

Somewhat similar differences in radiosensitivity are found in stationary-phase cultures of many strains of *E. coli* when they have grown in natural media, depending on whether glucose was present or absent.[5] Radioresistant cells obtained from glucose-containing media have also a large excess of DNA per cell as compared with those grown in the same medium with no added glucose. Such resistant cells, however, do not exhibit the imbalance with regard to other macromolecules.

A property common to the balanced and unbalanced situation is the excess amount of DNA per cell, and a question of some importance is what role the excess DNA plays in the observed radioresistance. Several lines of evidence obtained indicate that the excess DNA per se does not determine radiosensitivity:

1. Production of excess DNA precedes detectable increase in radioresistance in glucose cultures.
2. Some mutant strains of *E. coli* do not develop radioresistance even though the DNA per cell doubles.
3. Some strains of *E. coli* cannot express their radioresistance without postirradiation nutritional supplementation.

These findings suggest the importance of a repair system in which the excess DNA probably plays a role. The nature of such a system is under investigation. Two approaches are currently being

used: (1) measurement of the relative ability of radioresistant and sensitive cells to repair radiation-damaged phage and (2) determination of the relative capability of radioresistant and sensitive strains to incorporate genetic markers from a common donor strain, by use of the K-12 conjugation system. Both of these test systems are used to test the postulate that the resistant cells have an enzyme system for the repair process and that a process akin to somatic recombination is responsible for the repair process.

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6.2 INFLUENCE OF THE EXPERIMENTAL CONDITIONS ON THE OXYGEN EFFECT IN ORNL AND CSH STRAINS OF *ESCHERICHIA COLI* B/r

Roberto Masironi G. E. Stapleton

Introduction. – Preliminary findings of an unexpected influence of the environmental conditions on the oxygen effect in *Escherichia coli* were previously reported.[1] The results obtained along this line of evidence are summarized below.

The cells, suspended in suitable suspending medium, were x-irradiated while oxygen or nitrogen was bubbled through the suspensions. The experimental conditions checked for their possible influence on the survival of the irradiated cells and on the size of the oxygen effect were: type and concentration of the suspending medium (water, potassium or sodium phosphate buffer, isotonic NaCl, or isotonic KCl); temperature (ice bath or room temperature); mechanical preirradiation treatment (centrifugation and washing of the cells and subsequent dilution in the suspending medium or only dilution in the suspending medium); and degree of purity of the nitrogen used for removing the oxygen from the suspension.

With the exception of the last factor (no difference was found in the size of the oxygen effect when either nonpurified or purified nitrogen, or even helium, was used) all the other factors interfere with the oxygen effect.

Results and Discussion. – Table 6.2.1 shows data relating the percent survival of the ORNL and CSH strains of *E. coli* B/r for oxygen- and nitrogen-saturated suspensions of various salts. The concentrations are those determined to be the highest nontoxic levels for unirradiated cells. It is clear that:

1. No oxygen effect can be detected in high concentrations of NaCl, nor can any effect of temperature of irradiation be detected for oxygenated suspensions[2] of either strain. The ORNL B/r strain however shows a large effect of the temperature during irradiation for anaerobic suspensions.
2. Buffer suspensions of either strain, at either temperature, show an oxygen effect. A similar effect is observed for either the potassium or sodium phosphate buffer.
3. When the suspending solution is KCl a reverse oxygen effect is observed for the ORNL B/r strain.

Table 6.2.2 shows the influence of concentration of the various salts in the suspending medium for ORNL B/r. strain. Data are given for oxygenated and oxygen-depleted suspensions. When the cells are irradiated in oxygenated suspension, there is an increase in the survival per unit dose with increased salt concentration. Buffer suspensions show this increase in either oxygenated or oxygen-free condition. In other words an oxygen effect can be obtained at any buffer concentration. Anoxic NaCl suspensions, however, yield the same survival at all concentrations. Anoxic KCl suspensions, on the other hand, yield decreasing survival with increasing concentration.

The data so far reported are somewhat unexpected when one considers the large mass of literature compiled on the oxygen effect in various biological systems. Our review of the pertinent literature, however, reveals that most studies of the phenomenon in bacteria involved the use of phosphate buffer or buffered saline as the suspending media. Our results substantiate the earlier findings when this type of suspending media are used. It is remarkable indeed that minor

variations in the irradiation conditions produce large deviations from the oxygen effect as reported in much of the literature.

The effect of various salts as protectors in oxygen-containing suspensions accords with data reported for bacteriophage.[3] No such data are available for anoxic phage suspensions, and the data we present here for KCl suspensions are most unusual in exactly this condition. It is not clear how the various salts bring about their protection

Table 6.2.1. Effect of Suspending Medium[a] and Temperature on Survival and on the Size of the Oxygen Effect on ORNL and CSH Strains of *E. coli* B/r

Medium	Percent Survival at 31 Kilorads					
	Irradiated in Oxygen		Irradiated in Nitrogen		DMF[b]	
	Ice Bath	Room Temperature	Ice Bath	Room Temperature	Ice Bath	Room Temperature
ORNL Strain						
Water	0.06	0.06	12	4.0	2.2	1.8
Potassium-phosphate	3	3.2	40	40	2.2	2.2
Sodium-phosphate		4		40	2.2	
NaCl	3	3	22	3.1	1.6	1.0
KCl		2		0.04		0.55
CSH Strain						
Potassium-phosphate	0.06	0.06	2.0	2.0	2.2	2.2
NaCl	0.01	0.01	0.01	0.01	1.0	1.0

[a]The concentrations of the various salts are the highest concentrations determined to be nontoxic for unirradiated cells.

[b]DMF = ratio of dose in N_2 to that in O_2 to bring about equivalent inactivation.

Table 6.2.2. Effect of Different Concentrations of Different Suspending Media on Survival of ORNL Strain of *E. coli* B/r

Medium	Percent Survival at 31 Kilorads for Different Concentrations				
	0 (Water)	10^{-4} *M*	10^{-3} *M*	10^{-2} *M*	Isotonic
Irradiated in Oxygen at Room Temperature					
Buffer	0.06	0.2	1.0	2.0	3.2
Saline	0.06	0.1	0.4	1.0	3.0
KCl	0.06	0.08	0.2	0.7	2.0
Irradiated in Nitrogen at Room Temperature					
Buffer	3.0	3.0	3.3	5.0	40.0
Saline	3.0	3.1	3.0	3.2	3.1
KCl	3.0	2.0	0.6	0.15	0.04

or sensitization. It is not clear that the effect is one of dehydration as postulated previously.[3] Preliminary data suggest that the effect of the various salts is brought about before and during the irradiation since the survival is characteristic of the medium present during irradiation and independent of the nature of the postirradiation dilution medium.

It will be of some interest to determine whether such effects as reported here can be demonstrated in phage or in transforming DNA. Such experiments are planned for the future.

[1] R. Masironi and G. E. Stapleton, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 81.

[2] G. E. Stapleton and C. W. Edington, *Radiation Res.* 5, 39 (1956).

[3] C. S. Bachofer and M. A. Pottinger, *J. Gen. Physiol.* 37, 663 (1954).

6.3 GENETIC LOCUS AFFECTING CELL DIVISION AND RADIATION SENSITIVITY IN *ESCHERICHIA COLI* K-12

H. I. Adler Alice A. Hardigree

Mating experiments between a radiation-resistant high-frequency donor strain of *Escherichia coli* K-12 (HfrH) and a radiation-sensitive recipient (AB1899) have established that a gene (*lon*) closely linked to the T6 marker influences response to both ultraviolet (2537 A) and ionizing radiation. Mutation at the *lon* locus results in enhanced radiation sensitivity and a partial failure of the mechanism that forms the cross wall. Radiation induces the formation of long, nonseptate filaments in cells containing this mutation. The filaments grow for several hours after irradiation and synthesize nucleic acids at a near-normal rate. Nuclear division, observed by phase microscopy, also proceeds normally. The filaments do not form macroscopically visible colonies on nutrient agar. The frequency of filaments accounts quantitatively for the radiation inactivation of AB1899. Both ends of a filament gain the ability to produce cross walls and form macrocolonies on nutrient agar which contains pantoyl lactone or omega methyl pantoyl lactone. In this way the lactones completely remove the radiation sensitivity induced by mutation at the *lon* locus.

E. coli K-12 strains containing an alteration at the *lon* locus are usually observed to also produce a heavy mucoid capsule when grown in a glucose-synthetic medium. In order to establish whether the production of capsule is intimately related to radiation sensitivity and failure of the cross-wall-forming mechanism, we have isolated nonmucoid mutants of AB1899. One of these (AB1899NM) has been shown to have retained the radiation sensitivity of AB1899. It forms filaments that respond to pantoyl lactone in the same manner as those of AB1899. We have concluded that the effect of the *lon* locus on radiation behavior is not necessarily related to capsule formation. The genetic nature of the alteration in AB1899NM is currently under investigation.

6.4 EFFECTS OF NITROGEN MUSTARD ON GROWTH AND DIVISION OF *ESCHERICHIA COLI* K-12[1]

H. I. Adler Alice A. Hardigree

Using techniques previously described, we have been able to study the growth and division of single cells of *Escherichia coli* in considerable detail.[2] For example it has been observed that, for many strains, cell division is preceded by growth resulting in elongation at both ends of these rod-shaped bacteria. In *E. coli* B/r ORNL, unlike *E. coli* B ORNL and several K-12 strains, growth results in elongation at one end of the cell only.

After exposure to ionizing or ultraviolet (2537 A) irradiation, those *E. coli* strains in which elongation normally occurs at both ends of the cell produce long nonseptate filaments. We have now established that nitrogen mustard [methyl bis(β chloroethyl) amine hydrochloride] causes the formation of similar filaments. These filaments do not recover the ability to achieve normal cell division spontaneously and fail to give rise to macroscopically visible colonies. Cells in which the division mechanism has been damaged by nitrogen mustard can be stimulated to divide

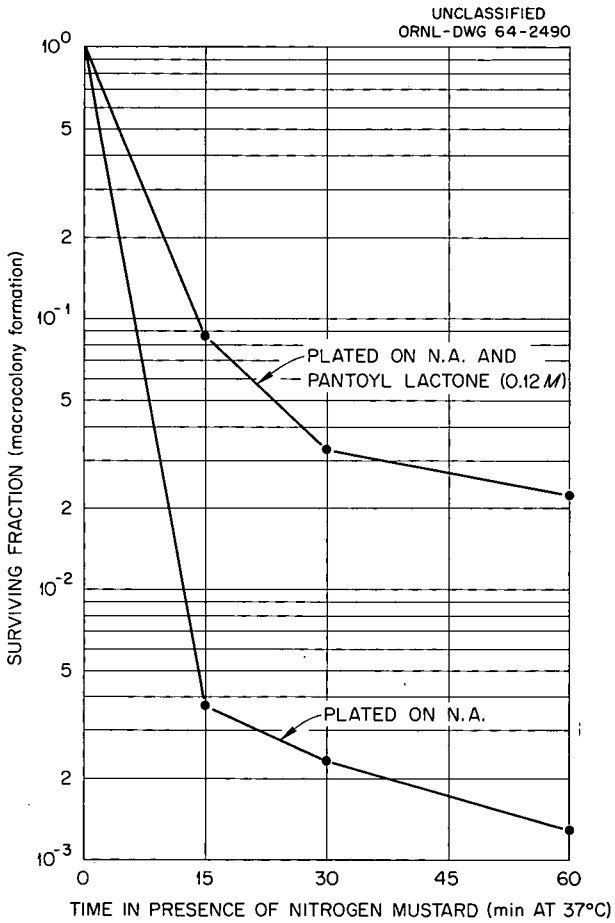

Fig. 6.4.1. Survival of *E. coli* K-12 AB1899 After Exposure to Nitrogen Mustard (5 mg/ml).

normally by incorporating pantoyl lactone (0.12 *M*) in the plating medium. The division-enhancing effect of pantoyl lactone on nitrogen-mustard-treated cells is reflected in formation of macrocolonies and can be observed in Fig. 6.4.1. The inactivation kinetics of *E. coli* K-12 by nitrogen mustard are similar to those previously reported.[3]

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

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

[3]R. Haynes and W. Inch, *Proc. Natl. Acad. Sci. U.S.* **50**, 839 (1963).

6.5 GENETIC ANALYSIS IN *ESCHERICHIA COLI* K-12 OF A DOUBLE MUTANT SENSITIVE TO RADIATION

D. E. Duggan H. I. Adler

Several different gene loci in the linkage group of *Escherichia coli* K-12 have been shown to be involved in determining the resistance of these organisms to radiations. Mutants more sensitive to ultraviolet than the parent have been isolated from *E. coli* K-12 AB-1886, itself an ultraviolet-sensitive mutant which has lost the mechanism responsible for "dark repair" of ultraviolet-induced thymine dimer lesions.[1] One of the mutants isolated was also more sensitive to x rays than the parent. These mutants, like the parent, form long filamentous cells after exposure to ultraviolet. The mutation can be distinguished from the filament-forming *lon* gene mutation[2,3] since it does not form mucoid colonies when grown on glucose-containing agar. Further, this mutant retains the ability to exhibit increased resistance to x rays (and reduced resistance to ultraviolet) when grown to stationary phase in a glucose-containing broth. This glucose effect is found in the parent but is absent in the *lon* gene mutation. Current experiments are designed to locate the newly mutated gene in the *E. coli* K-12 linkage group, with relation to previously described loci affecting response to radiation, and to determine the nature of the action responsible for the increased sensitivity to ultraviolet and x rays. This locus is not an early marker in HfrH, HfrC, AB311, or AB312, but among progeny from a cross with HfrH, 10% of those selected for His^+ Sm^R type were of the same resistance to ultraviolet as the parental AB-1886. None of the TL^+ Sm^R or Pro^+ Sm^R recombinants had received the resistance of the parental strain. These data suggest that the gene is located between the loci responsible for streptomycin resistance and for histidine synthesis. The *Sm-His* region of AB-1886 is not well marked; therefore two new markers, Ser/Gly^- and Lys^-, are being introduced into the sensitive mutant so that the mutated gene can be located more exactly. The Hfr strain AB-313 is being used to map the gene in this region of the linkage group. The AB-313 was cured of lysogeny to avoid difficulties with zygotic induction and with ultraviolet induction in progeny examined for radiation resistance.

[1]P. Howard-Flanders *et al.*, *Proc. Natl. Acad. Sci. U.S.* **48**, 2109 (1962).

[2]P. Howard-Flanders, *Genetics* **49**, 237 (1964).

[3]H. I. Adler and Alice A. Hardigree, *J. Bacteriol.* **87**, 720–26 (1964).

6.6 CHROMOSOMAL ABERRATIONS ASSOCIATED WITH MUTATIONS TO BACTERIOPHAGE RESISTANCE IN *ESCHERICHIA COLI*

Roy Curtiss III

Since *Escherichia coli* K-12 is sensitive to all the T phages and can be used to study gene transfer by conjugation, it was logical to study the genetic properties of phage-resistant mutants in this strain. Heretofore, no studies had been done with phage-resistant mutants isolated by selection with bacteriophage T3. Ten types of T3-resistant mutants were obtained, each type showing various combinations of resistance to the bacteriophages T4, T7, and λ. Two classes of T3-resistant mutants were isolated in which there was an obligate requirement for proline in order to obtain growth in minimal media ($/3,7,\lambda,pro_1^-$ and $/3,4,7,\lambda,pro_1^-$; the symbol / indicates resistance to). Many of the $/3,4,7$, $/3,4,7,\lambda$ and $/3,4,7,\lambda,pro_1^-$ mutants were unstable, changing to complete sensitivity to T4.

The $/3,4,7,\lambda,pro_1^-$ mutation in *E. coli* K-12 caused a break in the circular chromosome which eliminated the normal association in the inheritance of the outside markers leu^+ and lac^+. When sensitivity to T4 was regained, association in the inheritance of outside markers was restored and the resulting $/3,7,\lambda,pro_1^-$ mutation behaved genetically as a deletion. The $/3,7,\lambda,pro_1^-$ and $/3,4,7,\lambda,pro_1^-$ mutations caused inhibition of genetic recombination in regions adjacent to them, the formation of unstable partial diploid recombinants, and seemed to cause positive pairing interference.

One group of $/3,4,7,\lambda$ mutations were unlinked to the *leu* to *try* region of the bacterial genome. Other $/3,4,7,\lambda$ mutations in F^- bacteria destroyed the normal association in the inheritance of the outside markers lac^+ and gal^+; presumably by breakage of the circular chromosome, Hfr and F^+ strains with $/3,4,7,\lambda$ mutations at this locus were unable to conjugate and therefore a complete genetic analysis of the effects of this $/3,4,7,\lambda$ mutation could not be done.

7. Radiation Immunology

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7.1 GROWTH AND SENESCENCE OF THE PRIMARY ANTIBODY-FORMING POTENTIAL OF THE SPLEEN

Takashi Makinodan W. J. Peterson

Introduction. – Although the immune mechanism of an individual is presumed to play an essential role in aging, very little systematic data are available on the ability of an individual to respond to an antigen as a function of age. Studies were therefore carried out to determine the antibody-forming potential of mice of varying age groups and of the spleen of donor mice from varying age groups. The latter type of study was carried out to establish whether the change in the immune system of an individual with respect to age is due to a change in the functional lymphoid tissue and not to ancillary factors. This study, which was carried out over a period of 5 yr, confirms and extends our preliminary findings.[1]

Results. – The results showed that the maximum growth rate of the primary antibody-forming potential (PAFP) of the spleen occurred during the first month after birth. The mean doubling time during this period was ~4 days in contrast to ~14 days for spleen and body weights (see Fig. 7.1.1). The highest antibody-forming activity was

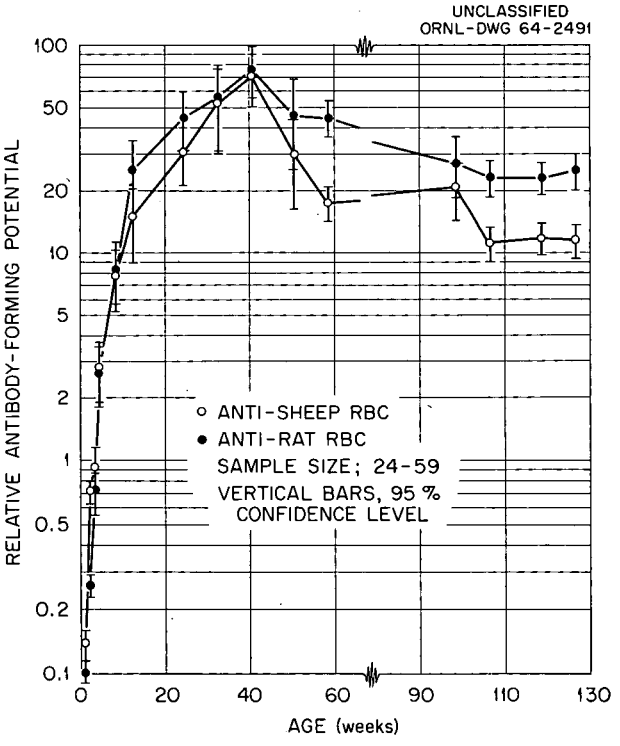


Fig. 7.1.1. Primary Antibody-Forming Potential of the Spleen as a Function of Age of Mouse.

obtained with cells from 40-week-old mice. The PAFP of the spleen increased 600-fold between 1 and 40 weeks of age. In contrast the body weight increased only eightfold and the spleen weight fourfold. After 40 weeks of age the PAFP of the spleen decreased gradually with age. Among the very old mice (~120 weeks of age) it was only one-fourth that of the spleen of 40-week-old mice. With improvement in the animal health program the PAFP of the spleen increased especially among the very old mice, and against a test antigen the host lacks any detectable amounts of natural antibodies. Evidence was obtained to show that the change in the PAFP of the spleen with age is due mainly to change in the number of potential antibody-forming cells. Furthermore it was shown that the change in the number of these cells with age is not due to immigration and emigration of these cells to and from the spleen.

Discussion. – These results show that the spleen, which constitutes about one-third of the total lymphoid mass of mice, is a suitable source to sample a unique population of cells reflecting the immune potential of an individual. These cells, which exist as a minority, are mainly undifferentiated, multipotent cells. These data strongly suggest that one fruitful approach to understand growth and senescence of a tissue is to study the undifferentiated stem cells capable of transforming into functional cells rather than the differentiated functional cells as done commonly.

[1]Takashi Makinodan and W. J. Peterson, *Proc. Natl. Acad. Sci. U.S.* **48**, 234 (1962).

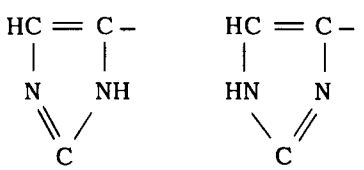
7.2 MECHANISM FOR ENZYME ACTION AND ITS RELEVANCE TO DIFFERENCES BETWEEN ANTIBODIES AND ENZYMES

R. A. Brown

Introduction. – In a recent article Elsasser[1] pointed out that the charge fluctuation on biological molecules, discussed in a paper by Kirkwood,[2] must be accompanied by a deformation of these molecules. He suggested that the deformations arising from charge fluctuations in an enzyme may strain the attached substrate in such a way as to favor the electronic transition from substrate to product.

Discussion. – If one is to accept this suggestion as a basis for discussing enzyme catalysis, one must first decide whether the relevant charge fluctuations are associated with the entire molecule or with the enzymatic site. Recent data[3] suggest that the enzymatic site is flexible and undergoes deformation upon the binding of the substrate. Therefore it seems more attractive to assume that the charge fluctuations are associated with the small area of the enzyme site.

Kirkwood's discussions of charge fluctuations involve the migration of protons among the ionizable groups of a number of amino acid residues. Such a mechanism would require a number of ionizable residues with appropriate ionization constants at the active site. An alternative possibility involves two tautomeric forms of the histidine imidazole group which are interconvertible by the migration of a proton:



Since the electronic structure is such that the net charge on the imidazole ring atoms will depend on the location of the proton,[4] migration of the proton would lead to a charge fluctuation. The resulting changes in electrostriction or hydrogen bonding would cause the site to be deformed.

The interaction of an antibody with its respective antigen and of an enzyme with its substrate appears to be a similar phenomenon, the only essential difference being the presence or absence of catalytic activity. If it is assumed that the catalytic activity results from deformation of a flexible binding site by a charge fluctuation in a histidine residue, it can be predicted that antibody sites would not be deformable and that histidine would not be a prominent feature of antibody binding sites. The kinetic studies of Day *et al.*[5] indicate that the binding of antigen by antibody does not result in a deformation of the binding site. Although the iodination experiments of Pressman and Sternberger[6] implicated tyrosine

and/or histidine in the binding sites of antibodies, the more recent work of Metzger *et al.*[7] suggests that only tyrosine is involved in the antibody binding site.

[1]W. E. Elsasser, *J. Theoret. Biol.* **3**, 164 (1962).

[2]J. G. Kirkwood and J. B. Shumaker, *Proc. Natl. Acad. Sci. U.S.* **38**, 855 (1952).

[3]D. E. Koshland, *Science* **142**, 1533 (1963).

[4]B. Pullman and A. Pullman, *Quantum Biochemistry*, Interscience, New York, 1963.

[5]L. A. Day, J. M. Sturtevant, and S. J. Singer, *J. Am. Chem. Soc.* **84**, 3768 (1962).

[6]D. Pressman and L. A. Sternberger, *J. Immunol.* **66**, 609 (1951).

[7]H. Metzger, L. Woofsy, and S. J. Singer, *Biochemistry* **2**, 979 (1963).

7.3 ASPECTS OF THE KINETICS OF ANTIBODY PRODUCTION BY MOUSE SPLEEN CELLS CULTURED IN VIVO

J. F. Albright T. W. Evans

Introduction. – We have postulated that primary and secondary antibody responses to a given antigen are initiated by cells which differ in their degree of differentiation.[1,2] Thus cells involved in primary response are multipotential, whereas the primed cells initiating secondary response are of relatively fixed destiny and adapted to the test antigen. It is possible, therefore, that the relation between antibody production and antigen dosage for the primed cells differs from that of the nonprimed cells and reflects the adaptation of the former to the test antigen. This possibility has been tested.

Methods and Results. – Spleen cells were obtained from either normal mice or mice primed optimally with the test antigen (Rrbc). Spleen cells from primed animals were suspended in three different concentrations; a single concentration of nonprimed cells was employed. Each preparation of cells was mixed in vitro with a series of different, tenfold dilutions of Rrbc. Aliquots of each final mixture of spleen cells and Rrbc were injected intravenously into a group of recipient mice. Antibody production was followed by daily bleedings of recipient mice and titration of the sera for hemagglutinins. The slope of the logarithmic phase of antibody production was found to be independent of spleen cell number or antigen dosage. Figure 7.3.1 shows the relative output (titer at the end of the logarithmic phase) of antibody per competent cell of the different spleen cell preparations. The number of competent cells is the initial number obtained from previous "all-or-none" assessment. It is apparent that the efficiency of antibody production per unit PC (potentially competent) cells is proportional to the antigen dosage and that the change in efficiency per given increase in antigen dosage is not dependent upon the total number of cells. Furthermore, the change in efficiency with antigen dosage is the same for both primed ("adapted") and nonprimed cells.

Discussion. – Our lack of understanding of the complex factors which regulate antibody production allows only tentative conclusions to be drawn from these data. The data indicate that the variation in efficiency of antibody production by PC cells with varying antigen dosage is independent of (1) whether or not the PC cells are primed, (2) the total number of PC cells, (3) the total number of spleen cells. The data focus attention on the need for thorough analysis of the initial events of PC-cell–antigen interaction.

[1]Takashi Makinodan and J. F. Albright, p. 99 in *Immunopathology*, Schwabe and Company, Basel, Switzerland, 1963.

[2]E. H. Perkins, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 113.

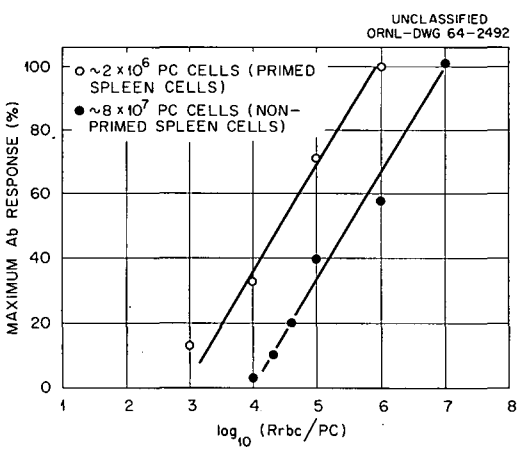


Fig. 7.3.1. Relative Total Antibody Production per Unit Potentially Competent Spleen Cells in Relation to Antigen Dosage.

7.4 DESTRUCTION OF RED BLOOD CELL ANTIGENS BY PERITONEAL PHAGOCYTIC CELLS

E. H. Perkins

Introduction. – Phagocytosis has been implicated to be an essential primary event in antibody formation. Many believe that these calls after digestion of antigenic material transfer the "antigenic information" to other cell types capable of supporting antibody formation. Three types of studies were carried out to test this possibility using peritoneal phagocytes.

Results. – In vitro studies were first carried out by culturing 10^7 peritoneal phagocytic cells with 10^7 sheep red blood cells. The immunogenic activity was assessed by injecting aliquots of this reaction mixture with 10^7 primed spleen cells into lethally irradiated recipient mice. This method permits us to detect as low as 10^4 RBC equivalents. It was found that 6 and 12 hr after in vitro incubation 90 and 99% of the immunogenic units, respectively, were destroyed. Diffusion chamber studies were then carried out by culturing 10^6 RBC in the presence or absence of 10^7 peritoneal phagocytic cells and with or without 10^7 primed spleen cells. The results showed that, although this number of peritoneal phagocytic cells were incapable of forming detectable amounts of antibody in the presence of the test antigen, their presence interfered with the antibody-forming capacity of the spleen cells. Finally, antibody response studies with intact normal mice demonstrated that as the dose of antigen becomes limiting (10^7 or 10^6 RBC) the intraperitoneal route of injection is markedly inferior to the intravenous route.

Discussion. – These results strongly suggest that peritoneal phagocytic cells destroy red blood cell antigens and are, in fact, competing for these same antigens with potentially competent antibody-forming cells. However, these studies do not exclude the possible role in antibody formation of phagocytic cells located strategically among potentially competent antibody-forming cells in the spleen, lymph node, and other lymphoid tissue.

7.5 SUPPRESSION OF APPEARANCE OF ANTIBODY-FORMING CELLS WITH SUPRAOPTIMAL DOSES OF ANTIGEN

Toshihiko Sado Carol J. Chadwick

Introduction. – It is known that competent cells do not appear when nonprimed lymphoid cells are stimulated with either suboptimal or supraoptimal doses of antigen. Previous studies in this laboratory[1] showed that suboptimal anti-RBC responses can be induced in a similar manner even with use of lymphoid cells from primed donors. However, we do not know whether the suboptimal response is due to decrease in the efficiency of antibody synthesis per functional cell or to decrease in the number of functional cells. In an attempt to have a better insight into this problem, we have used soluble BSA antigen in this study. This permits us to assess immunohistochemically the number of competent antibody-forming cells in relation to the amount of antibodies synthesized.

Method. – Spleen cell donors were either primed adult mice or albino rabbits. Generally 24×10^6 primed spleen cells were cultured in 0.1-μ-porosity diffusion chambers with varying doses of BSA ranging from 5×10^{-6} mg to 30 mg per chamber. Anti-BSA titers were determined by Stavitsky's passive hemagglutination method. The number of antibody-containing cells per chamber was estimated by multiplying the number of cells per chamber by the percentage of antibody-containing cells determined with Coon's immunohistochemical method.

Results and Discussion. – The results showed that a response of ~5% of the maximum was obtained with an antigen dose either 100-fold more than or less than the optimal dose. It was found that the optimal dose of BSA is $\sim 5 \times 10^{-2}$ mg per 24×10^6 primed spleen cells. As shown in Fig. 7.5.1, the number of antibody-containing cells per chamber is proportional to the anti-BSA titer of the chamber fluid. We can conclude therefore that suppressed secondary antibody response with excess dose of antigen is due not to decrease in the efficiency of functional cells but to decrease in the number of functional cells. These results

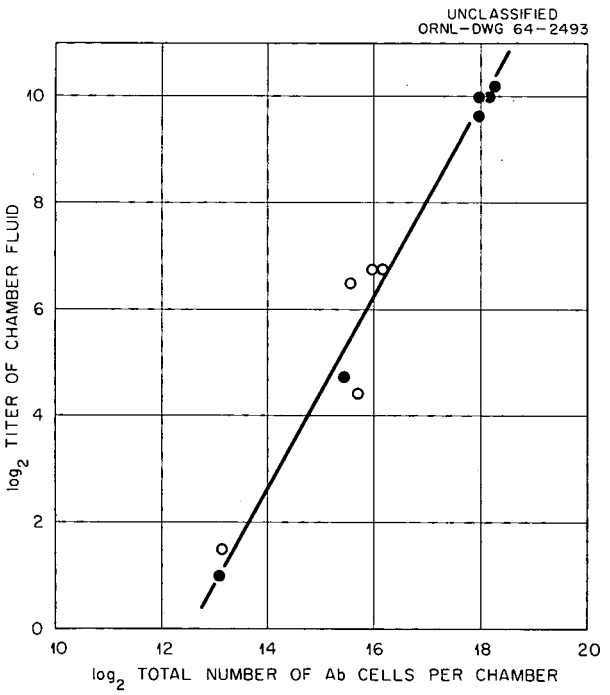


Fig. 7.5.1. Relation Between Antibody Titer and Total Number of Antibody-Containing (Fluorescent-Positive) Cells.

indicate that on the cellular level the problem of immunological responsiveness and unresponsiveness resides not only on the multipotent, primary cells but also on the unipotent, secondary cells, both of which require antigenic stimulation before they are transformed into functional cells.

[1]Immo Hoppe, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963,* ORNL-3498, pp. 114–15.

7.6 FATE OF THORACIC DUCT LYMPHOCYTES AFTER ANTIGENIC STIMULATION

Paul Nettesheim

Introduction. – Although it is known that proliferating blast cells give rise to functional antibody-forming cells, it is not known what cells, if any, give rise to blast cells. The hypothesis that small lymphocytes dedifferentiate into blast cells which then differentiate into functional antibody-forming cells has been in vogue for the past few years in spite of the fact that definitive evidence is lacking. Experiments were therefore carried out to test whether most, if not all, of the blast cells in the latent phase of the immune response arise from small lymphocytes through dedifferentiation. Thoracic duct lymphocytes of inbred rats were cultured in 0.1-μ-porosity diffusion chambers together with the test antigen. In the control group the lymphocytes were cultured in the absence of the test antigen.

Results and Discussion. – Although no difference in the number of cells per chamber was seen between the experimental and control groups, the number of blast cells per chamber in the experimental group was higher than that of the control group from one to six days after antigenic stimulation. Six days after antigenic stimulation these cells constituted 60% of the total population. In contrast these cells did not appear in detectable quantity in the absence of antigenic stimulation (see Fig. 7.6.1). Having obtained this information, H^3-thymidine (H^3Tdr) was then injected

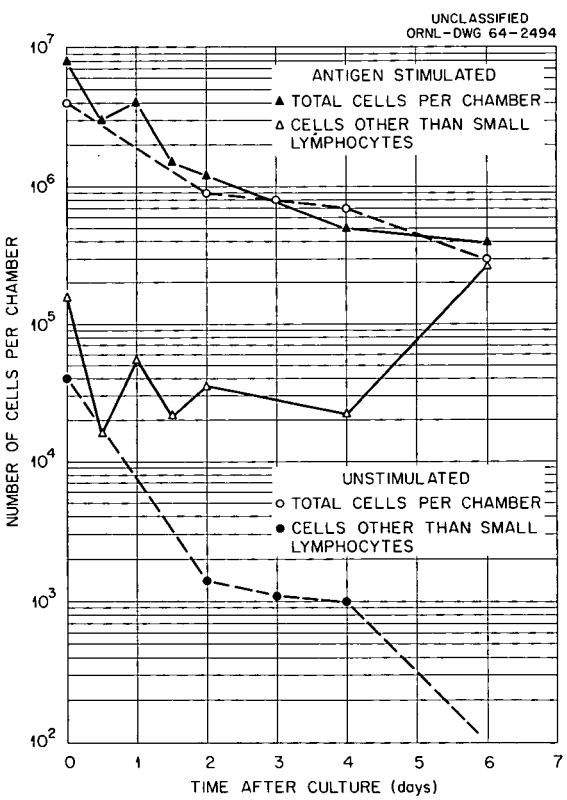


Fig. 7.6.1. In Vivo Culture of Thoracic Duct Lymphocytes.

either 0, 12, 24, 36, 48, 60, 72, or 84 hr after culture. Chamber samples were obtained 1 and 12 hr after each pulse injection of H^3Tdr on the assumption that there will be no more than two cell divisions within 12 hr. Labeled small lymphocytes were not detected 1 hr after pulse labeling. It follows that if small lymphocytes were dedifferentiating into blast cells then the ratio of unlabeled blast cells to labeled blast cells 12 hr after pulse labeling is expected to be significantly larger than that 1 hr after pulse labeling. No increase in this ratio was seen.

Conclusion. – These data do not support the hypothesis that when stimulated by an antigen small lymphocytes dedifferentiate into blast cells. However they do suggest that blast cells arise from DNA-synthesizing large and medium lymphocytes.

7.7 CELLULAR CHANGES DURING EMBRYONIC SPLENOMEGALY

Frank Seto

Introduction. – Although the histological changes of the host embryo spleen during the splenomegaly response have been described, no quantitative cytokinetic analyses have been performed. Experiments were initiated for this purpose. The increases in total spleen cell number in normal and experimental embryos were reported earlier.[1] The results of tritiated thymidine (H^3Tdr) labeling studies and differential cell counts will be described in this report.

Methods. – The normal proliferative capacity of embryonic spleen was estimated from spleen cell counts of embryos, 8 to 19 days of incubation, which had been pulse-labeled with H^3Tdr and sacrificed 1 and 6 hr later. One group of experimental 15-day-old embryos was injected with about 10^6 H^3Tdr-labeled donor spleen cells, sacrificed at 12-hr intervals, and spleen imprints and smears of cell suspensions prepared. A second group of recipient embryos was pulse-labeled two days after nonlabeled donor cell injection, sacrificed at 10-hr intervals, and similar spleen-cell preparations made. The spleen imprints and cell smears were processed for autoradiography or stained with Wright-Giemsa for differential counts.

Results and Discussion. – In the normal embryos, prior to the 12th day of incubation when the spleen growth is logarithmic, over 35% of the cells may be pulse-labeled. After the 12th day the spleen growth rate declines and the percent cells which are pulse-labeled decreases to about 20%. The control embryonic spleen growth correlates well with change in proliferative capacity. On the other hand the percent labeling in the experimental embryos, two days after donor cell injection when the spleen is in the log phase of splenomegaly, is about 35% – significantly higher than the 20% labeling in control embryos of the same age. Associated with the

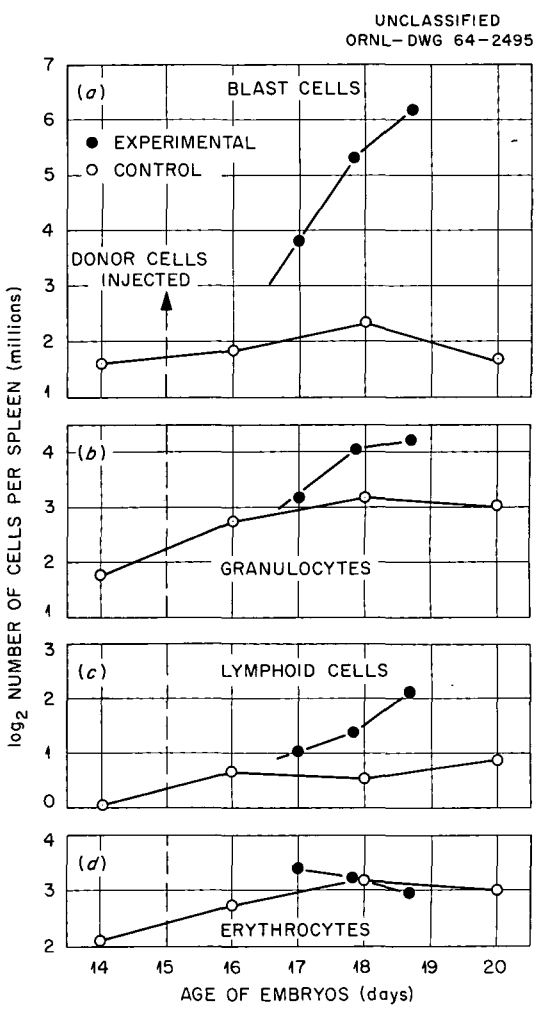


Fig. 7.7.1. Change in Number of Spleen Cell Types in Control and Experimental Embryos.

enhanced proliferative activity and dramatic increase in total spleen cells, the number and relative proportion of blast cells increased tremendously (Fig. 7.7.1*a*). The increase in the other cell types was less impressive (Fig. 7.7.1*d*). When the spleens of host embryos which received labeled donor spleen cells were examined, about 1% of the cells were of donor origin in the first 6 hr and the number increased during the subsequent 12-hr intervals. Despite the increase in donor cells the data indicate that the initial inoculum size and proliferation rate are insufficient to account for splenic enlargement. The results from these experiments and from previous x-irradiation studies[1] indicate that the host embryo not only possesses sufficient proliferative potential but does in fact contribute significantly to the splenomegaly.

[1]Frank Seto, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 116–17.

8. Tissue Culture

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8.1 STUDIES ON THE FREQUENCY OF X-RAY-INDUCED CHROMATID ABERRATIONS INDUCED AT DIFFERENT TIMES OF THE CELL CYCLE

J. G. Brewen

Introduction. – It has been known for some time that, depending on the stage of the cell cycle occupied by the cells at the time of irradiation, there are both qualitative and quantitative differences in the x-ray-induced chromosomal aberrations observed at metaphase. Qualitative differences are determined by whether or not the chromosome has replicated at the time of irradiation. The quantitative differences, however, require more complex explanations. Some of the differences, that is, the difference between G_1 and S cells, can be explained by a bias in scoring at the subsequent metaphase (see Wolff[1] for details). The difference in frequencies observed in cells irradiated in S as compared to cells irradiated in G_2 (G_2 cells having more aberrations per unit dose) has been explained by spatial relations between chromosomes, that is, the site hypothesis (see Wolff for details). The present experiments were performed to determine the cause of the apparent increase in sensitivity of G_2 cells to irradiation.

Materials and Methods. – Lateral roots of *Vicia faba* beans were used for all experiments. Mitotic synchronization was attained by treatment with 5-amino-uracil (5-AU), a pyrimidine analog known to block DNA synthesis. When the treatment with 5-AU ceases, the cells, which are all in the S stage of the cell cycle, proceed through S, G_2, and mitosis in a synchronized fashion. At various intervals (see Tables 8.1.1 and 8.1.2) after removal from 5-AU, groups of four beans were pulse-labeled with tritiated thymidine for 15 min. Two of these beans were then irradiated with 25 r of x rays. In all of the roots treated in this fashion the cells reached mitotic prophase $9\frac{1}{2}$ hr after removal from 5-AU, at which point metaphases were collected by colchicine treatment for 2 hr.

Results. – Tables 8.1.1 and 8.1.2 summarize the results obtained in these experiments. Three hours after removal from 5-AU the majority of the cells were in the S stage, that is, were capable of incorporating ^{3}H-thymidine into their chromosomes. This population of cells slowly progressed from S to G_2, as can be followed by the labeling pattern observed in Fig. 8.1.1. Six hours after removal from 5-AU, all of the cells were in G_2. Simultaneously, as the labeling index drops with increasing time after removal from 5-AU, the frequency of chromatid aberrations increases until 5 to 6 hr. At this point the frequency of chromatid aberrations levels off and remains constant until $8\frac{1}{2}$ hr after removal, at which time there is a slight increase. At 9 hr a very large decrease in

Table 8.1.1. Controls at Various Times After Removal from 5-AU

Time (hr)	Number Scored	Percent Deletions	Percent Isochromatid	Percent Exchanges	Percent Total Breaks	Percent Heavily Labeled
3	150	0.0	2.0	2.0	6.0	70.0
3½	150	0.0	0.7	1.3	3.3	56.6
5	150	0.0	2.7	1.3	5.3	9.7
6	150	0.0	0.7	2.0	4.7	5.3
7	150	0.0	1.3	1.3	4.0	9.0
7½	150	0.0	0.0	2.0	3.0	0.0
8	150	0.0	0.7	1.3	3.3	1.0
8½	150	0.7	1.3	1.3	4.7	0.0
9	150	0.0	0.7	1.3	3.3	1.0
Total	1350	0.07	1.1	1.6	4.3	

Table 8.1.2. Aberrations Induced at Various Times After Removal from 5-AU

Time (hr)	Number Scored	Percent Deletions	Percent Isochromatid	Percent Exchanges	Percent One-Hit	Percent Breaks	Percent Heavily Labeled
3	300	1.0	5.3	2.0	6.3	10.3	71.0
3½	200	1.0	4.0	4.0	5.0	13.0	58.0
5	300	1.7	5.7	7.3	7.3	22.0	11.7
6	300	0.3	5.3	9.7	5.7	25.0	6.0
7	300	3.0	5.0	8.0	8.0	24.0	
7½	300	4.7	3.3	8.3	8.0	24.7	0.3
8	300	3.0	6.0	8.7	9.0	26.3	0.0
8½	300	3.7	4.7	11.0	8.3	30.3	0.0
9	250	1.6	2.4	5.6	4.0	15.2	1.0

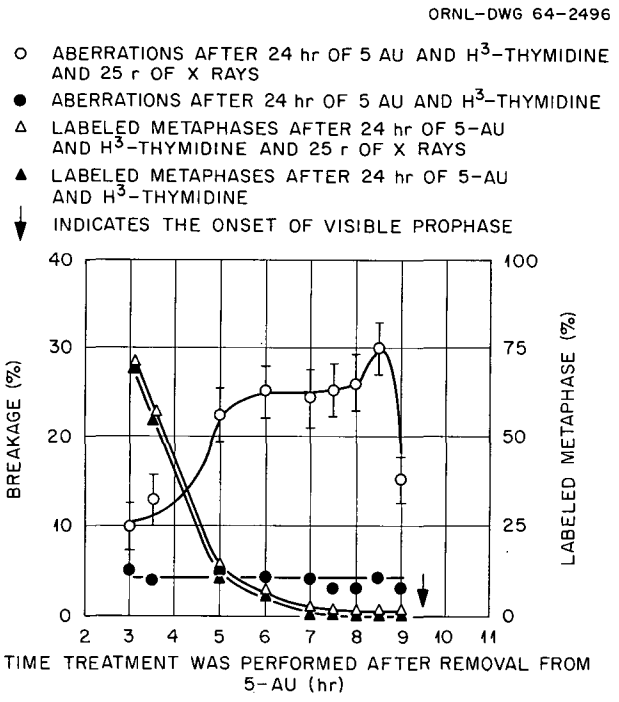

Fig. 8.1.1. The Relation Between Frequency of Aberrations and Time of Cell Cycle During Which the Treatment Was Given.

the aberration yield is observed. The rise in the aberration yield between 3 and 5 hr agrees with the time that the cells are passing from S to G_2.

Discussion. – If the sensitivity of the chromosome strand to ionizing radiations increases as the cell passes from S to G_2, one would then expect that all types of chromatid aberrations would increase when the cell enters G_2. It is observed, however, that the frequency of one-hit aberrations, that is, terminal deletions and isochromatids, remains reasonably constant throughout S and G_2, but that the frequency of two-hit aberrations, that is, exchanges, increases at the time the cell enters G_2. These data are interpreted, then, as indicating that the chromosome strand does not increase in its sensitivity as the cell enters G_2, but that the number of sites within which an exchange can form increases as the cell enters G_2, and that this increase in site number accounts for the apparent increase in sensitivity of G_2 cells to x rays.

[1]S. Wolff, *J. Cellular Comp. Physiol.* **58,** 151 (1961).

8.2 MORPHOLOGICAL ALTERATION OF SMALL LYMPHOCYTES IN HUMAN LEUKOCYTE CULTURES

Hiroshi Yamamoto

Introduction. – In phytohemagglutinin-stimulated tissue cultures of human leukocytes, it has been shown that most of the cells which undergo mitosis are large mononuclear cells.[1] Since many more of these cells are present in dividing cultures than in the leukocyte population of fresh blood, it is evident that some other type of leukocyte is "converted" into a large mononuclear cell during the premitotic phase of the culture. In vivo, the lymphocyte is known to transform into monocytic or other types of cell in the connective tissues. In order to see whether the large mononuclear cell in phytohemagglutinin-stimulated cultures is similarly derived from the lymphocyte, leukocyte cultures were studied with both the light microscope and the electron microscope.

Materials and Methods. – Adult human peripheral leukocytes were cultured with mixture 199 supplemented with human serum and phytohemagglutinin. Every 6 hr after initiation, the cells from one of a series of replicate cultures were fixed with 2% potassium permanganate, embedded in epoxy resin, sectioned on a Porter-Blume microtome, and examined with an RCA EMU3E electron microscope. Thicker sections (1 to 2 μ) were cut and stained with Geimsa for light microscopy, and differential cell counts and measurements of size were made.

Results and Discussion. – In differential cell counts (Fig. 8.2.1) the numbers of polymorphonuclear granulocytes and of small lymphocytes decreased during the first 36 hr of culture life. Large mononuclear cells, on the other hand, increased in number during this period. No mitotic cells were seen until 48 hr after initiation of the cultures, which agrees with the autoradiographic data of Bender and Prescott.[1]

The large mononuclear cells showed prominent nucleoli, most of which had clear nucleolonema; the average nucleolar number was about 1.1. Both cellular and nuclear size are increased over those seen in human peripheral lymphocytes. With electron microscopy, the nucleoli in the large mononuclear cells fixed with potassium

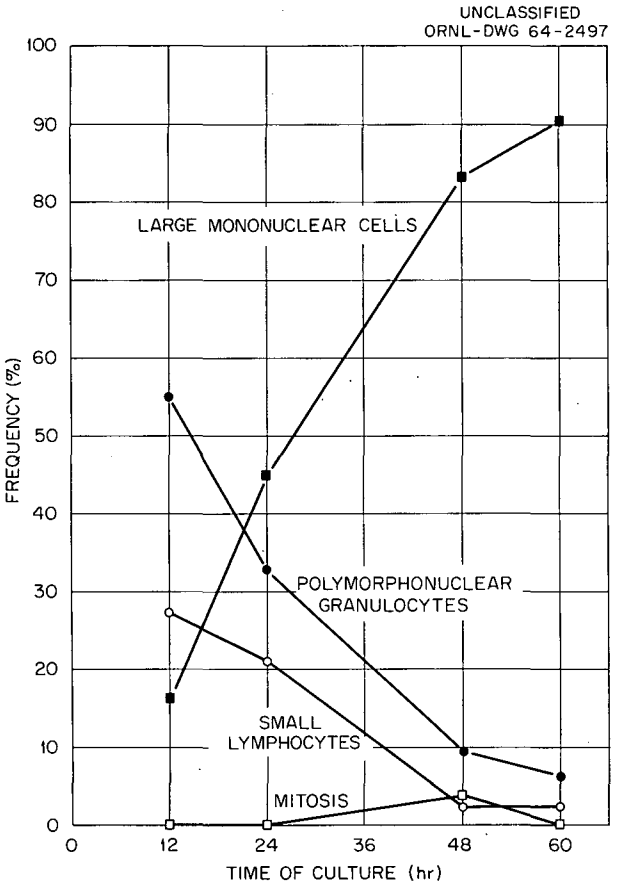

Fig. 8.2.1. Differential Cell Counts of Human Leukocytes in Phytohemagglutinin-Stimulated Cultures.

permanganate consisted of two zones: a peripheral reticular zone and a central, less electron-dense, zone. At the surface of the peripheral reticular zone, perinucleolar chromatin covered the nucleoli as a thin electron-dense layer. In human small lymphocytes, the chromatin usually appears as clumped, electron-dense structures. In the large mononuclear cells from the cultures, however, the chromatin appeared as less clumped structures, dispersed as granules throughout the nuclei. The Golgi complexes, which occur more frequently, and are more developed in the large mononuclear cells than in the mature lymphocytes, are located near the nucleus. The tubular structures of the endoplasmic reticulum are also more abundant in the large mononuclear cells than in mature lymphocytes. Microbody-type lysosomes and multivesicular bodies are also more frequent in the large mononuclear cells. In all of these characteristics, the large mononuclear cells from the cultures are similar to lymphoblasts.

Even when the phytohemagglutinin stimulus was removed after only 6 hr, the large mononuclear cells still appear and mitoses are seen 48 hr after culture initiation.

In view of the decrease in the numbers of small lymphocytes and granulocytes in phytohemagglutinin-stimulated cultures and the concomitant increase in the numbers of lymphoblast-like cells, and of the appearance of cell types apparently intermediate between the small lymphocyte and the lymphoblast, it is concluded that phytohemagglutinin stimulates the conversion of small lymphocytes into lymphoblast-like cells, which subsequently undergo mitosis. It appears that the "dedifferentiation" is determined after as little as 6 hr of contact with the stimulating agent and that it will then continue without further contact with the agent.

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[1]M. A Bender and D. M. Prescott, *Exptl. Cell Res.* **27**, 221 (1962).

8.3 ABERRATION RATES INDUCED BY 2.5-Mev NEUTRON IRRADIATION OF HUMAN BLOOD IN VITRO

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E. B. Darden[1]
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Introduction. We have undertaken a study of neutron RBE values and yield kinetics for human somatic chromosomal aberrations, in order to provide a basis for radiation dose estimates from peripheral leukocyte chromosomal aberration frequencies in cases of whole-body exposure during criticality accidents.[3] We have previously reported data on aberrations induced in human leukocytes by 250-kv x rays and by 14-Mev neutrons.[4,5] We have now completed a similar study of the effects of 2.5-Mev neutrons.

Table 8.3.1. Aberrations Induced by 2.5-Mev Neutrons in Human Blood Irradiated in Vitro

Dose (rads)	Cells Scored	Cells with $2n \neq 46$	Chromatid Aberrations	Chromosome-Type Aberrations					
				Deletions	Rings	Dicentrics	Exchanges	Deletions (%)	Rings and Dicentrics (%)
Control	500	54	15	8	0	1	1	1.6	0.2
15	500	38	10	23	3	9	14	4.6	2.4
30	550	42	3	56	5	31	10	10.2	6.5
45	500	60	6	66	8	76	34	13.2	16.8
60	446	46	5	85	9	42	37	19.1	11.0

Method. – Freshly drawn whole human blood was irradiated in nylon chambers specially designed to make the blood sample conform to the isodose contours surrounding our neutron source. After irradiation, the leukocytes were separated, cultured, fixed, mounted, and stained by the techniques used in our previous studies.[4,5] The experiments were repeated five times, using a different blood donor each time. The neutrons were generated by the D,D reaction, using a Cockcroft-Walton accelerator. Only relatively low dose rates could be achieved; the average was about 1.5 r/min.

Results and Discussion. – Table 8.3.1 shows the pooled results for the five experiments. The frequencies of abnormal chromosome counts and of chromatid-type aberrations are not different from those observed in other experiments. The frequency of chromosome-type deletions in the controls is somewhat higher in the 2.5-Mev neutron experiments than is usual. Six of the eight deletions seen occurred in cells from one donor; other unirradiated cultures from this donor show the usual low frequency (0.25%) of chromosome deletions. The individual 2.5-Mev neutron experiments show somewhat more variability than we have seen in other studies. This is particularly evident in the values for dicentric yield at 45 and 60 rads. The increased variability is thought to be due to the rather critical geometry required for the irradiations because of the low intensity of the 2.5-Mev neutron flux. A simplified calculation from the data in Table 8.3.1 yields a coefficient of 0.26×10^{-2} deletions per cell per rad for 2.5-Mev neutrons. If this is compared with the coefficient of deletion production by 250-kv x rays,[4,5] the RBE value for 2.5-Mev neutrons is about 2.6, the same as was found for 14-Mev neutrons.[5] The yield of ring and dicentric chromosomes with 2.5-Mev neutrons shows the expected linear relation to dose. The calculated coefficient of aberration production is 0.23×10^{-2} rings and dicentrics per cell per rad. Since the kinetics of production of these aberrations are different from those for x and gamma rays, it is impossible to calculate an RBE value for ring and dicentric production by 2.5-Mev neutrons.

[1]Pathology and Physiology Group.

[2]Biophysics.

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

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

[5]P. Carolyn Gooch, M. A Bender, and M. L. Randolph, *Proceedings of the Symposium on Biological Effects of Neutrons*, IAEA, Upton, New York, 1963 (in press).

9. AEC-NASA Space Biology Program

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9.1 EFFECTIVENESS OF HIGH-ENERGY PROTONS ON VARIOUS BIOLOGICAL MATERIALS

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This portion of the Space Biology Program was designed to assess the relative biological effectiveness of protons over the spectrum of energies encounterable in sustained flight. The energies used ranged from 22 to 750 Mev, covering most of the important energy range in the Van Allen radiation belts and overlapping to some extent the expected energies from solar flares.

A number of biological test systems were chosen, ranging from the measurement of inactivation and mutation in *Escherichia coli* to measurement of production of chromosome aberrations in leukocytes of human blood samples. The experiments are as follows:

1. (*a*) Measurement of the RBE of the various proton energies for inactivation and mutation in *E. coli*. (*b*) The influence of oxygen on these processes and in experiments on chemical protection by sulfhydryl compounds (e.g., MEA) on proton inactivation of the bacteria.

2. Measurement of inactivation and mutation at the adenine locus in *Neurospora crassa*. The fine structure of this locus in this species is now known in some detail, and this knowledge lends itself well to studies of RBE of radiations as a function of LET.

3. Measurement of the relative effectiveness of the various energy protons in production of lethality in spermatogonia and oocytes of mice. The quantitative aspects of this phenomenon are well characterized and lend themselves well to RBE studies.

4. Measurement of chromosome aberrations in human leukocytes. An "in vitro" irradiation system has been developed which permits irradiation of small volumes of the investigators' blood, and later by incubation of leukocytes separated

therefrom by routine tissue culture techniques to quantitate the aberrations per cell as a function of dose. This system is well adapted to RBE determinations.

All of these systems are known to respond to variations in LET by change in the RBE. It is well established from previous work in this Laboratory and elsewhere that the systems do not respond in the same way. For example, bacterial inactivation efficiency decreases with increasing LET above 1000 Mev-cm^2/g. Most of the other systems show increased efficiency with increased LET, but the quantitative relations are not well established. Since the bacterial inactivation system shows essentially an RBE of 1 up to LET of ~1000 Mev-cm^2/g, it served a twofold purpose in the experiments: (1) a sort of biological dosimeter and (2) the RBE for mutation induction and the response to aerobiosis and anaerobiosis could be established as a function of LET.

Radiations. – The base-line radiation was 250-kvp x rays, and all RBE determinations are based on the effect per unit dose with this radiation. For all the biological systems a large backlog of information is available from experiments with this radiation. In addition, to assess any effect of transportation on mice used in some of the experiments, a parallel series of animals was irradiated at the various sites with 250-kvp x rays.

Particle Accelerators. – External beams of various energies of protons were obtained at the various following research sites:

22-Mev protons – ORNL 86-inch cyclotron

130-Mev protons – Synchrocyclotron, Harvard University

430-Mev protons – Synchrocyclotron, Enrico Fermi Institute of Nuclear Science, University of Chicago

750-Mev protons – Synchrocyclotron, Lawrence Radiation Laboratory, University of California

Heavy ions – Heavy-Ion Linear Accelerator, Lawrence Radiation Laboratory, University of California

With the exception of the ORNL cyclotron, these aforementioned facilities are routinely used for biological and biomedical investigations and the dosimetry is well worked out. Careful determination of the homogeneity of the 22-Mev proton beam was made by measurement of the darkening of glass and photographic film. From the measured proton flux and use of the accepted values for energy loss in the biological sample, it was possible to calculate the dose in rads. In later experiments thin metaphosphate glass rod dosimeters and LiF thermoluminescent dosimeters were incorporated into the physical setup.

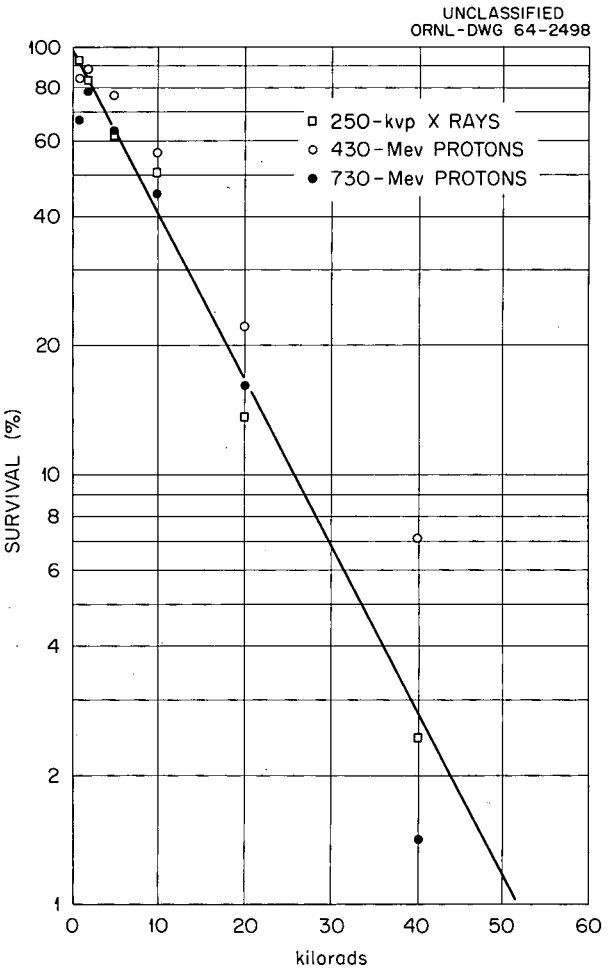

Fig. 9.1.1. Inactivation of Heterokaryotic Conidia of Neurospora.

Results. – To date bacterial experiments have been performed with protons of the various energies. Human blood was irradiated with 130-, 430-, and 750-Mev protons. Use was also made of 100- and 50-Mev protons in one experiment. *Neurospora* conidia have been irradiated with 22-, 430-, and 750-Mev protons. Mice have been irradiated for germ-cell inactivation only with 130- and 750-Mev protons. The results available for the various systems studied to date are shown in Tables 9.1.1–9.1.3 and Figs. 9.1.1 and 9.1.2. Also included in Table 9.1.1 are data for bacterial

inactivation with high-LET carbon ions which demonstrate reduction in RBE at high LET; also as expected the ratio of sensitivity of aerobic vs anaerobic cells is decreased. The apparent decrease in this ratio with 22-Mev protons may be due to lack of sufficient anaerobiosis, and more experiments on this point are needed and are under way. The data for chromosome aberrations in human white blood cells for 50- and 100-Mev protons are incomplete and will be reported later. The data for inactivation of *Neurospora* conidia are shown in Fig. 9.1.2. Although there are some deviations from the standard x-ray inactivation curve, the proton curves are not significantly different from the x-ray curve. The number of mutants per 10^6 surviving conidia per unit dose for 430-Mev protons and 250-kvp x rays are shown in Fig. 9.1.2. Although irradiations have been made with 750- and 22-Mev protons, the data for mutation frequencies have not as yet been tabulated. The data for inactivation of mouse spermatagonia and oocytes by x rays and 130-Mev protons are shown in Table 9.1.3. Scoring of the tissues from the 750-Mev proton irradiations is not as yet complete.

It is obvious from the data so far available from experiments with the various protons for the several biological materials studied that no systematic deviation from RBE of 1 has been obtained. Although the systems respond to LET by changes of RBE, over the LET range studied, 2.5 to 25 Mev-cm^2/g, no change in efficiency is observed in bacterial, fungal, or mammalian cell systems for inactivation, mutation, or chromosome damage.

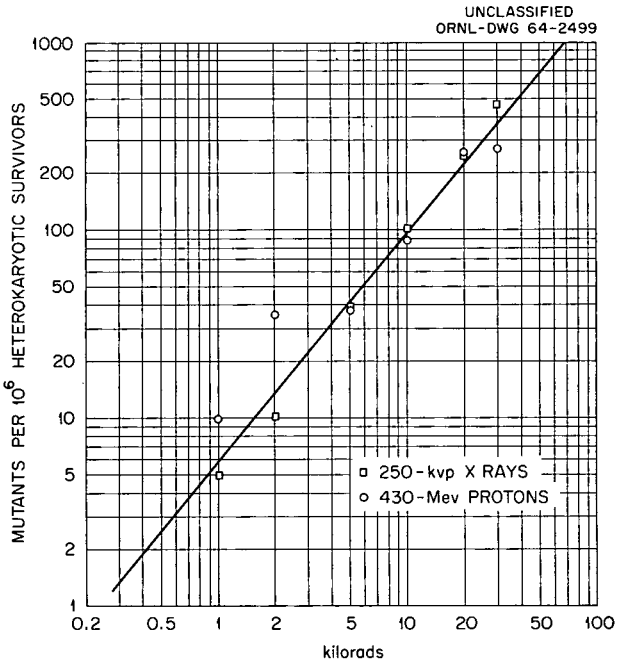

Fig. 9.1.2. Mutation Induction on the *ad-3* Region of Neurospora.

Table 9.1.1. Bacterial Inactivation and Mutation

Radiation	LET (Mev-cm^2/g)	Inactivation Coefficient, $1/D_0$ (kilorads)		Ratio Aerobic/Anaerobic	Mutation Coefficient, Revertants per Survivor per Kilorad	
		Aerobic	Anaerobic		Proline	Galactose
					$\times 10^{-10}$	$\times 10^{-10}$
750-Mev protons	~2.5	0.14	0.043	3.0	10	20
430-Mev protons[a]	~3.0	0.20			10	30
250-kvp x rays	~5.0	0.125	0.040	3.1	12	20
130-Mev protons	~7.0	0.13	0.041	3.1	10	20
22-Mev protons	~25.0	0.13	0.062	2.1		
100-Mev carbon ions	~2000.0	0.11	0.078	1.4		

[a]The data obtained with the 430-Mev proton beam are somewhat less reliable than the others because the uniform beam area was smaller and the dosimeter system was not cross-calibrated with the other radiations.

Table 9.1.2. Production of Chromosome Aberrations in Human White Blood Cells

Radiation	Number of Cells Scored	Coefficient of Aberration Production[a]		RBE
		Deletions	Rings and Dicentrics	
		$\times 10^{-2}$	$\times 10^{-5}$	
430-Mev protons	500	0.08	0.42	0.7–0.8
250-kvp x rays	~2000	0.11	0.55	1.0
130-Mev protons	500	0.11	0.60	1.0–1.1

[a]Number of the type of aberrations per cell per rad for deletions and per square rad for rings and dicentrics. The former aberrations are one-hit and the latter are two-hit events.

Table 9.1.3. The Ratio of Experimental to Control Observed 72 hr After Irradiation

Dose (rads)	Ratio for Cells of Type Indicated					
	Type A Spermatogonia[a]		Type B Spermatogonia[b]		Preleptotene Spermatocytes[c]	
	130-Mev Protons	X Rays	130-Mev Protons	X Rays	130-Mev Protons	X Rays
5	0.85	0.89	1.013	0.93	0.99	0.93
10	0.97	0.75	0.96	0.89	1.005	0.85
25	0.73	0.60	0.81	0.71	0.72	0.64
50	0.50	0.34	0.43	0.17	0.33	0.13
100	0.32	0.20	0.08	0.02	0.01	0.00
200	0.18	0.12	0.01	0.00	0.00	0.00

[a]Irradiated and scored as type A spermatogonia.

[b]Irradiated as type A, scored as type B spermatogonia.

[c]Irradiated as late type A and early intermediate spermatogonia, scored as preleptotene spermatocytes.

9.2 DEVELOPMENT OF A RADIATION–SPACE-FLIGHT INTERACTION EXPERIMENT FOR THE GEMINI-III MANNED SPACE FLIGHT

M. A Bender P. Carolyn Gooch
Sohei Kondo

Introduction. – At the request of NASA, the Biology Division has undertaken the design and development of an experiment to test for possible unpredicted effects of the radiation encountered during space flight and for possible interactions between radiation and other space-flight parameters, such as weightlessness. A number of observations of biological systems subjected to space flights have suggested the possibility of such unpredicted effects. The Biology Division experiment is to be carried on the Gemini-III manned space flight, scheduled for the last quarter of 1964. The experiment will measure the frequencies of various types of chromosomal aberrations in a series of samples of whole, heparinized human blood exposed to a known quantity and quality of radiation during the "weightless" phase of the space flight, in unirradiated "in-flight" controls, and in a similar series of control samples exposed simultaneously on the ground. Both pre- and post-flight analyses will be made of material from the astronauts selected for the Gemini-III mission.

Experimental Development. – Because of the complicated nature of the experiment itself, and because it must be packaged in a way which, while not endangering the Gemini-III mission in any way, will provide the proper environment for the blood samples, the largest part of the work associated with the experiment consists of the design, fabrication, and testing of the required "hardware."

On January 11, 1964, only two weeks after the Biology Division was asked for a conceptual design of the experiment, we were asked to deliver a "hard" mockup of the experimental package as soon as possible to the NASA Manned Spacecraft Center. Arrangements were made with the Oak Ridge Y-12 Plant to do the required design, fabrication, and physical testing of the device and with the ORNL Isotopes Division to undertake the development and fabrication of the isotopic radiation sources. On January 27, 1964, only 16 days after it was first requested by the NASA, two models of the experimental device were delivered to the Manned Spacecraft Center. One of the designs was selected for inclusion on the Gemini-III mission. Figure 9.2.1 shows the major features of this device.

Three-milliliter samples of blood from each of two donors are located in each of five blood sample holders. In the original model these holders were constructed of machined nylon, Mylar, and aluminum. More recent work has yielded a stronger and more economical design, constructed of molded epoxy resin and glass fiber. The sample holders are closed, after sterile filling, with nylon screw plugs, which contain silver metaphosphate fluoroglass dosimeters. The radiation sources are composed of aluminum supports carrying platinum foil disks on which the required amount of P^{32} has been plated, covered by a thin "flash" of gold. The sources are arranged in a stack in such a way that when the blood sample holders are inserted between alternate source plates the blood samples are irradiated from both sides at a nominal dose rate of 10 rads/min. Four of the pairs of blood samples will receive doses of 50, 100, 150, and 200 rads. Different doses are given by pairs of sources of different activity. The fifth pair of samples, located at one end of the stack, is the control and does not receive any radiation. The whole array is housed in an aluminum box approximately $3\frac{3}{8} \times 3\frac{3}{8} \times 1\frac{1}{4}$ in. overall. Prior to activation of the device the blood samples to be irradiated are held to one side of the stack of sources; at the proper point in the space flight the stack of blood samples is inserted into the stack of sources by one of the astronauts. After the required doses have been accumulated, the samples are again withdrawn from the source array. Time-temperature and time-actuation records are made by an electrooptical instrument package included within the device. Ambient radiation dose is measured by silver metaphosphate fluoroglass dosimeters of larger volume than those incorporated in the blood sample chamber plug screws. The device's aluminum housing is sealed after assembly by field welding. The actuation device operates manually through a vacuum seal and firmly locks the blood samples in the "irradiate" and "non-irradiate" positions. The entire device, as delivered to the Manned Spacecraft Center, including the blood samples and instrument package, weighs 321 g.

All components of the experimental device have been subjected to most of the parameters associated with the launch, orbital, and recovery phases of the space flight in ground tests. The device has with-

stood most of the pressures, vacuum, vibration, and *g* loading tests required by the NASA. Complete qualification tests for the Gemini spacecraft will be performed at a later date. The blood sample holders have successfully passed biological tests, and satisfactory chromosome preparations have been made from blood samples held for 24 hr at room temperature in prototype sample holders. A prototype P^{32} source has been fabricated, and preliminary radiation measurements indicate that the experimental device is radiologically "safe."

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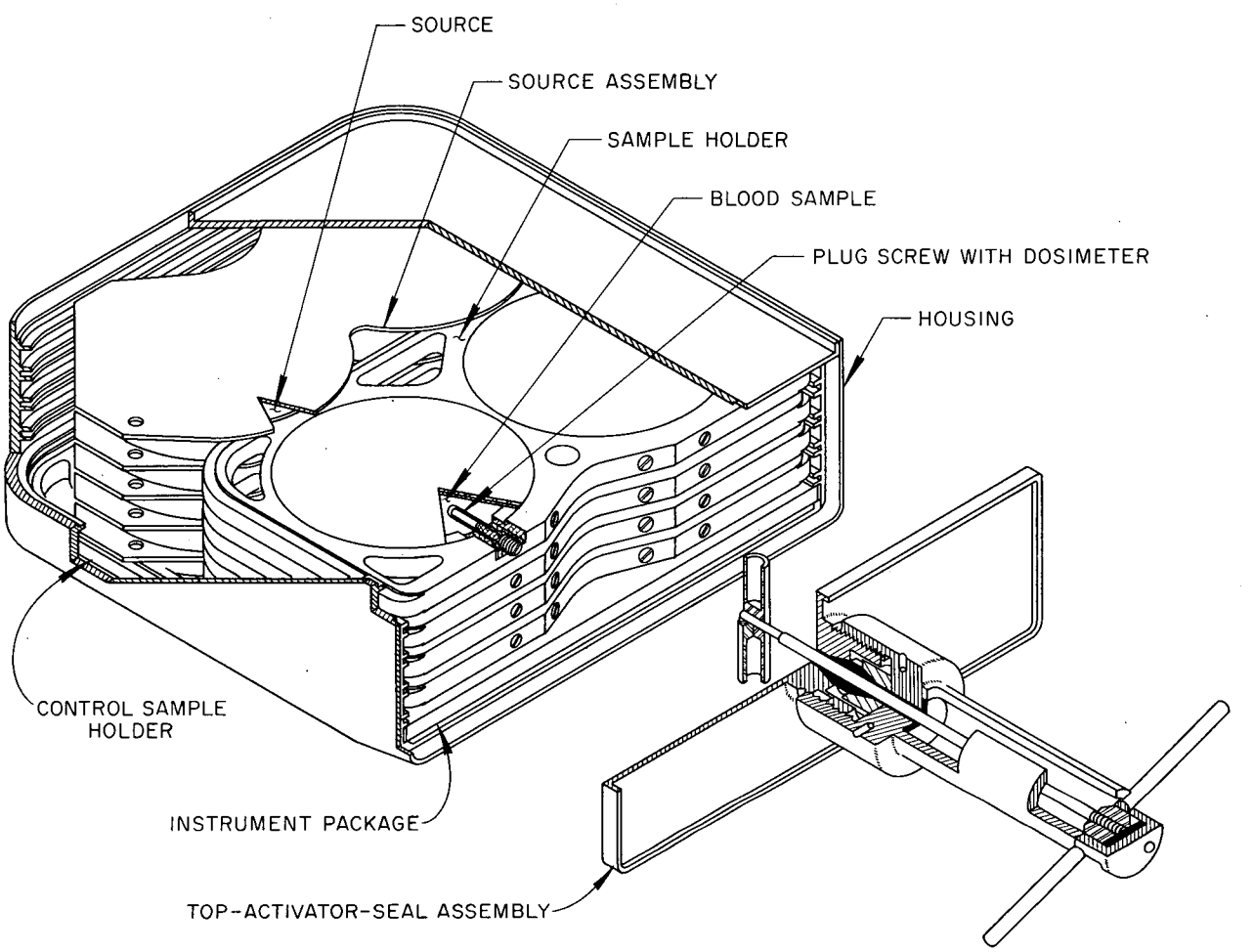

Fig. 9.2.1. Prototype Device for Blood Irradiation Experiment for the Gemini-III Manned Space Flight.

10. Mammalian Recovery

C. C Congdon[a]	
Secondary Disease (10.1–10.6) C. C Congdon D. C. Swartzendruber W. H. McArthur[b] Stanfield Rogers[b] Vu Thi Suu[c,d] Frances E. Hacker Kathleen H. Harrington Alberta P. Henley M. G. Hanna, Jr.[e]	**Hemopoietic and Lymphatic Stem Cells (10.9)** Joan W. Goodman Mysa C. Mathews **Bone Marrow Tissue Culture (10.10)** Henrianne G. Willard
Recovery in Vitro (10.7–10.8) Phan The Tran[c] T. W. McKinley, Jr.	**Animal Testing Program (10.11)** M. L. Simmons Everline B. Wright

[a]Dual assignment.
[b]Consultant.
[c]Visiting investigator from abroad.
[d]Agency for International Development.
[e]ORINS Fellow.

10.1 MORTALITY FROM SECONDARY DISEASE IN HOMOLOGOUS BONE MARROW CHIMERAS[1]

C. C Congdon

Introduction. – The general plan of this study was given in an earlier report that outlined the problem of the variable 90-day mortality from secondary disease.[2]

Methods and Results. – Thirty-, sixty-, and ninety-day mortality in 587 $1C3F_1$/Cum male and female mice exposed to 950 r before an intravenous injection of $B6D2F_1$/Cum bone marrow was determined in a one-half replicate of a 2×2^3 factorial experiment with center points. Four factors were examined for their effect on mortality: bone marrow cell dose, day of bone marrow injection, age of donor cells, and sex of recipient. Some of the mice surviving 90 days were tested and were shown to have donor red blood cells. Thirty-day mortality was greatest with a low dose of fetal or newborn blood-forming cells taken from liver just before or just after birth. Ninety-day mortality was not affected by sex of the recipient, bone marrow dose (10 to 50 million cells), or age of donor cells (0 to 90 days). However, there was more variability in the 90-day mortality than would be expected from binomial variation. Figure 10.1.1 shows an extreme example of the variable mortality in two groups given apparently identical treatment. One group has over 70% mortality at 90 days, while the other showed less than 10% deaths.

Discussion. – Further studies are needed to see if interaction among these four factors might have an influence on 90-day mortality, as well as to estimate the variance in a single kind of experimental situation. Particular attention needs to be

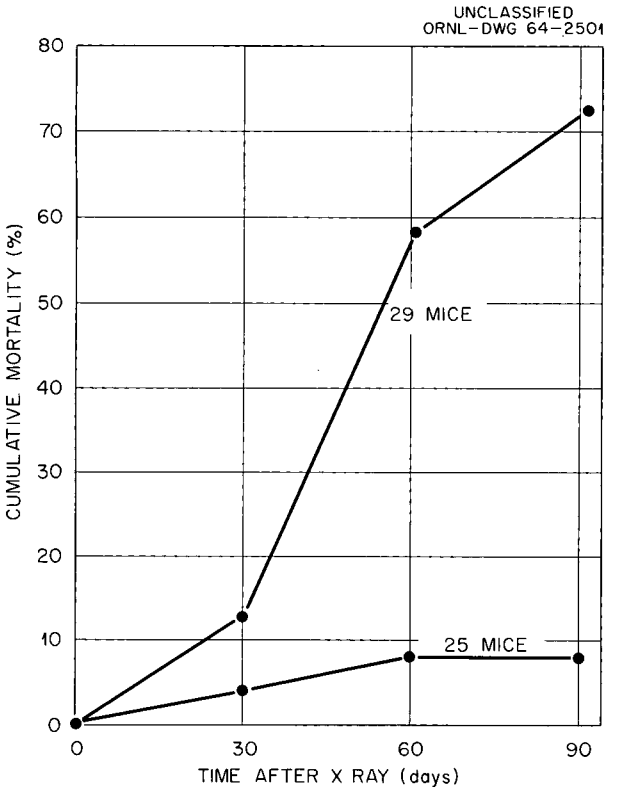

Fig. 10.1.1. Two Groups of $1C3F_1$ Mice Exposed to 950 r and Given 30×10^6 $B6D2F_1$ Bone-Marrow Cells on Day $1\frac{1}{2}$ After Irradiation.

paid to cage effects, because some of the data indicated that death from secondary disease was more common in some cages than in others.

[1]In collaboration with M. A. Kastenbaum and D. A. Gardiner, Mathematics Division, ORNL.

[2]C. C Congdon, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963,* ORNL-3498, p. 91.

10.2 NITROGEN BALANCE IN RADIATION CHIMERAS[1]

W. H. McArthur

Lethally irradiated mice can recover if they are injected with normal bone marrow cells. If the injected cells are genetically different from the irradiated host, the animal survives the radiation syndrome, but may die later because of a secondary disease process not related to the acute effects of irradiation. Secondary disease is a consequence of an immunological reaction between the grafted cells and the host animal.[2] The body weight loss and the failure of hair to grow in foreign marrow chimera suggest the mice are suffering from a metabolic starvation. Kretchmar and Congdon[3,4] have studied the biochemistry of the metabolic disease process on the assumption that it involves a disturbance in protein metabolism. Food intake of homologous-marrow-treated mice is adequate and comparable to that of normal or isologous-marrow-treated animals. We have previously reported results that confirmed the similarity of food intake among normal and isologous- or homologous-marrow-treated irradiated mice. We have also reported that mice of these three groups had comparable degrees of positive nitrogen balance. The results reported here are from more recent experiments. They show positive nitrogen balance, but no gain in body weight among the homologous chimeras. These experiments consisted of 24 mice as follows: 8 normals, 8 isologous, and 8 homologous. These data cover experiments from day 1 through day 90.

Table 10.2.1 shows that for the first 30 days both isologous- and homologous-marrow-treated groups were in positive nitrogen balance and retained more nitrogen than did normal control mice. Weight gain of isologous-marrow-treated animals was the same as that of normal mice. Animals given homologous marrow gained little or no weight.

Table 10.2.2 shows that animals treated with isologous marrow maintain a normal rate of gain in body weight through 90 days, but retain significantly more nitrogen than normal animals.

Table 10.2.1. Average Weight Gain and Nitrogen Balance for the Period 10–30 Days of Normal Mice and of Mice Given 950 r Total-Body Irradiation and Isologous or Homologous Bone Marrow Cells

Group	Weight Gain (g/day)	Nitrogen Balance (mg/hr)
Normal	0.06	0.55
Isologous	0.06	0.70
Homologous	0.02	0.73

Table 10.2.2. Average Weight Gain and Nitrogen Balance for the Period 10–90 Days of Normal Mice and of Mice Given 950 r Total-Body Irradiation and Isologous or Homologous Bone Marrow Cells

Group	Weight Gain (g/day)	Nitrogen Balance (mg/hr)
Normal	0.06 ± 0.012	0.34 ± 0.05
Isologous	0.05 ± 0.016	0.67 ± 0.12
Homologous	0.009 ± 0.007	0.72 ± 0.14

Animals given homologous marrow fail to gain weight, but retain twice as much nitrogen as normal mice. Both marrow-treated groups show the same degree of increased positive nitrogen balance.

We speculate that nitrogen is directed into some abnormal compartment or compartments in animals with secondary disease, and that this is related in some way to the immunologic conflict between grafted homologous cells and the host animals.

[1]In collaboration with A. L. Kretchmar, ORINS, Medical Division, and C. C Congdon.

[2]I. S. Urso, C. C Congdon, and R. D. Owen, *Proc. Soc. Exptl. Biol. Med.* **100** (1959).

[3]A. L. Kretchmar and C. C Congdon, *Am. J. Physiol.* **200**, 102 (1961).

[4]A. L. Kretchmar and C. C Congdon, *Exptl. Molecular Pathol.* **2**, 277 (1963).

10.3 TOTAL NITROGEN RECOVERY IN THE NORMAL MOUSE

A. L. Kretchmar[1]

Introduction. – An experiment to determine whether significant shifts occur in nitrogen content of various compartments of mice with secondary disease after marrow transplantation is being carried out because previous work indicated that the animals were in positive nitrogen balance despite weight loss (see Chap. 10.2). It was postulated that nitrogen was being stored in some abnormal form in these animals. The results to be reported here are from a pilot experiment on normal mice to serve as a check on the method of nitrogen recovery and to indicate the nitrogen levels for normal mice.

Methods. – The animals were divided into three groups, six mice in each group. The mice in group A were killed at the beginning of the experiment to give total nitrogen recovery values to be expected among animals of the colony before the experiment in metabolism cages was started. Group B mice were kept in metabolism cages for 48 hr, and nitrogen lost or gained was calculated as "nitrogen balance" from the total nitrogen intake minus the nitrogen excreted in urine and feces. Group C mice were similarly caged, but were maintained in the metabolism cages for two consecutive 48-hr periods (total of 96 hr), with separate computations of "nitrogen balance" being made for both the first and second 48-hr periods. Group B and C mice were killed at the end of the metabolic periods, and nitrogen content of the various compartments was determined.

Results. – The data are summarized in Table 10.3.1. The mean nitrogen content for the different compartments is given in milligrams for each of the three groups. For group A the standard error of the mean is also listed. The same relative error was found in the data for groups B and C and is, therefore, not included. The difference in groups B and C from A are also shown (B – A and C – A). Nitrogen balance is shown for group B, and the result indicates that animals of this group lost an average of 46 mg of body nitrogen during the 48-hr period. The differences in column 3 (15.3 mg) and column 5 (18.8 mg) between B and A show that this loss occurred from the abdominal viscera and carcass. The recovery, 34.1 mg (15.3 + 18.8 mg) vs 46 mg, is satisfactory in view of the standard error of the means. Group C animals were in negative balance for the first 48 hr (–47.6 mg) as shown for group B, but, during the second 48-hr period, four of the six mice were in positive balance, so that in these animals some of the loss was regained. The differences C – A, shown in columns 3 and 5, indicate that recovery was quantitatively greater in the abdominal viscera compartment relative to the carcass. These results are what would be expected from the known lability of the nitrogen in abdominal viscera. Since muscle protein is quantitatively the chief body protein reserve, though generally less rapidly mobilized than protein of the abdominal viscera, the loss from carcass that occurred in group B and C mice

Table 10.3.1. Nitrogen Content (mg) of Various Compartments in Normal Mice

Group	Nitrogen Content (mg)				Nitrogen Balance (mg)
	Blood and Thoracic Viscera	Abdominal Viscera	Skin, Tail, and Head	Carcass	
A	31.4 ± 1.9	102.3 ± 4.2	224.8 ± 6.5	232.8 ± 10.3	
B	30.8	87.0	224.0	214.0	−46
B − A		−15.3		−18.8	
C	31.1	96.5	226.0	220.0	
C − A		−5.8		−12.8	

was also expected. It is concluded that nitrogen lost or gained during a metabolism study is satisfactorily recovered by the techniques employed, and that shifts in nitrogen content of the order of 10–20% should be detectable by this kind of study.

[1]Medical Division, Oak Ridge Institute of Nuclear Studies, collaborative research with Mammalian Recovery Group.

10.4 MURAMIDASE ACTIVITY IN KIDNEYS, SPLEEN, AND BONE MARROW OF MICE CARRYING RAT BONE MARROW GRAFT

Vu Thi Suu

Introduction. – As shown in previous reports,[1,2] a marked increase in muramidase[3] activity was found in kidneys of homologous bone marrow chimeras. In the present report, a study of muramidase activity in kidneys, spleen, and bone marrow of mice carrying rat bone marrow grafts was undertaken during the first 30 days after irradiation and bone marrow treatment.

Methods and Results. – Male mice (LAF_1/Cum), 12 to 20 weeks of age, were used as recipients. Male Sprague-Dawley rats were used as donors of foreign bone marrow. Four groups of animals were studied. One group was normal control, the second group was x-ray control (950 r), the two other groups were mice irradiated (same dose) and treated with 40×10^6 isologous bone marrow cells (IBM) or 100×10^6 rat bone marrow cells (RBM). Bone marrow cells were given within 4 hr after irradiation. The animals were weighed, then decapitated at 3, 7, 9, 12, 15, 18, 20, 24, 28, and 30 days after irradiation and bone marrow treatment. Normal and irradiated mice were killed at the same intervals as control groups. Organs were rapidly excised, weighed, then wrapped in aluminum foil and frozen on dry ice until assayed. The tissues were allowed to thaw 30 min at room temperature, then the tissues were homogenized, centrifuged, and the muramidase activity determined as described in a previous work.[4]

Figure 10.4.1 is a plot of muramidase activity (referred to a standard enzyme preparation) in kidneys of normal mice, x-irradiated mice (950 r), mice x-irradiated and treated with isologous bone marrow (IBM), or rat bone marrow (RBM). The figure shows a marked increase in muramidase activity in kidneys of mice carrying rat bone marrow grafts. This increase begins at day 9. It reaches maximum values at day 15 and during the third and fourth weeks.

Table 10.4.1 shows the muramidase activity in the spleen from these same groups of mice. Elevation of the enzyme activity in the spleen of the RBM group occurred at the same time as that seen in the kidney.

Muramidase activity in the marrow of the RBM group was high at days 15 and 18.

Discussion. – These results demonstrate that increased muramidase activity is present in the kidney, spleen, and bone marrow of the mouse receiving a transplant of rat marrow. Similar findings were made in the earlier work on homologous bone

Table 10.4.1. Muramidase Activity in Spleen of Normal Mice, of Irradiated Mice (950 r), of Mice Irradiated and Treated with 40×10^6 Isologous Bone Marrow Cells (IBM), or 100×10^6 Rat Bone Marrow Cells (RBM)[a]

Time After Irradiation (days)	Muramidase Activity (μg per gram of spleen)			
	Normal Control	X-Ray Control	IBM	RBM
3	12 (7)	0 (18)[b]	0 (12)[b]	0 (12)[b]
7	34 (8)	0 (16)[b]	41 (8)	12 (8)
9	12 (7)	0 (12)[b]	19 (8)	48 (10)
12	18 (8)	0 (7)[b]	12 (8)	32 (8)
15	18 (8)		19 (8)	71 (7)
18	19 (8)		25 (8)	56 (8)
20	14 (7)		28 (8)	75 (8)
24	13 (8)		19 (8)	45 (7)
28	19 (8)		13 (8)	89 (7)
30	6 (7)		6 (7)	42 (8)

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

[b]"0" refers to no decrease in optical density after $2\frac{1}{2}$ min incubation of extract with substrate.

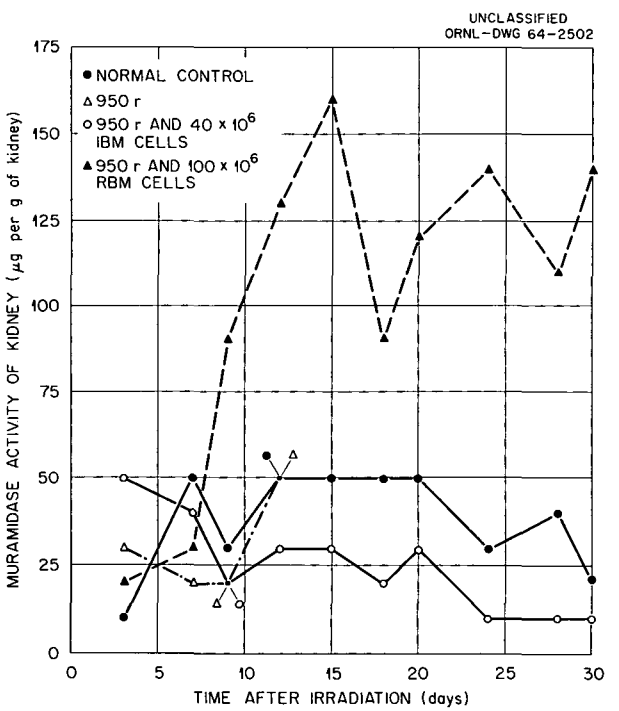

Fig. 10.4.1. Muramidase Activity in Kidneys of Mice. Each point is the average of values from 12 to 16 kidneys pooled per group.

marrow chimeras. The biochemical significance of changes in muramidase activity is not known. Neither is it known whether the muramidase is of donor or host origin.

[1]Vu Thi-Suu, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp. 78–79.

[2]Vu Thi Suu, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 87–89.

[3]Name recommended by Commission on Enzymes of International Union of Biochemistry to replace "lysozyme" (E.C.3.2.1.17).

[4]Vu Thi Suu, C. C Congdon, and A. L. Kretchmar, *Proc. Soc. Exptl. Biol. Med.* (in press).

10.5 ELECTRON MICROSCOPIC AUTORADIOGRAPHY OF SPLEEN GERMINAL CENTER CELLS AFTER ANTIGENIC STIMULATION

D. C. Swartzendruber

Introduction. – There is general agreement that cells which synthesize antibodies arise from a family of cells which multiply and differentiate as a response to antigenic stimulation. The plasma

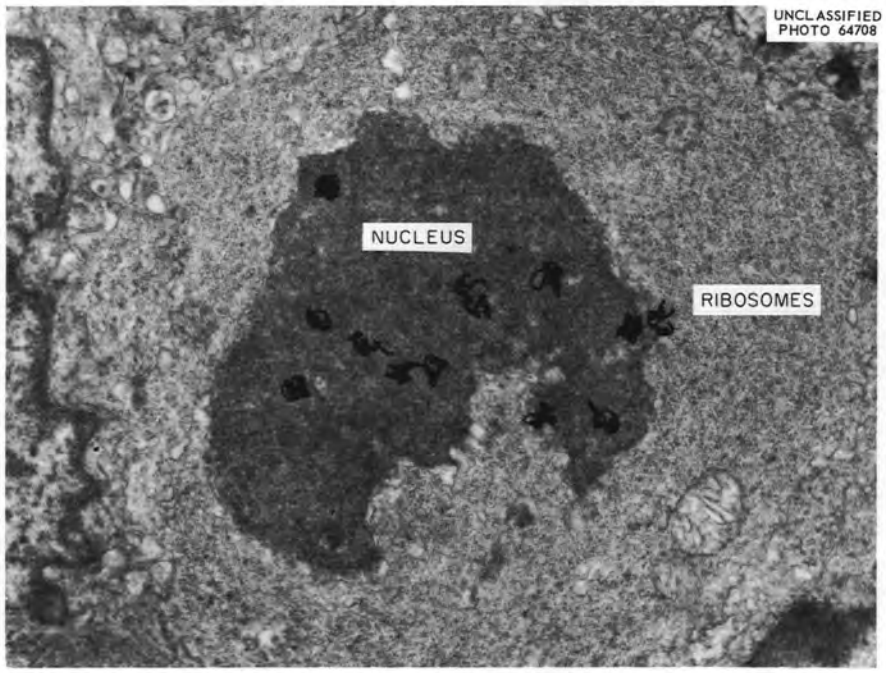

Fig. 10.5.1. Electron Microscopic Autoradiograph of a Labeled Germinal Center Cell 4 hr After Injection of Sheep Erythrocytes. Twelve or thirteen silver grains are over the nucleus, which is probably in early prophase. Abundant free ribosomes fill the cytoplasm. 15,000X. Reduced 29%.

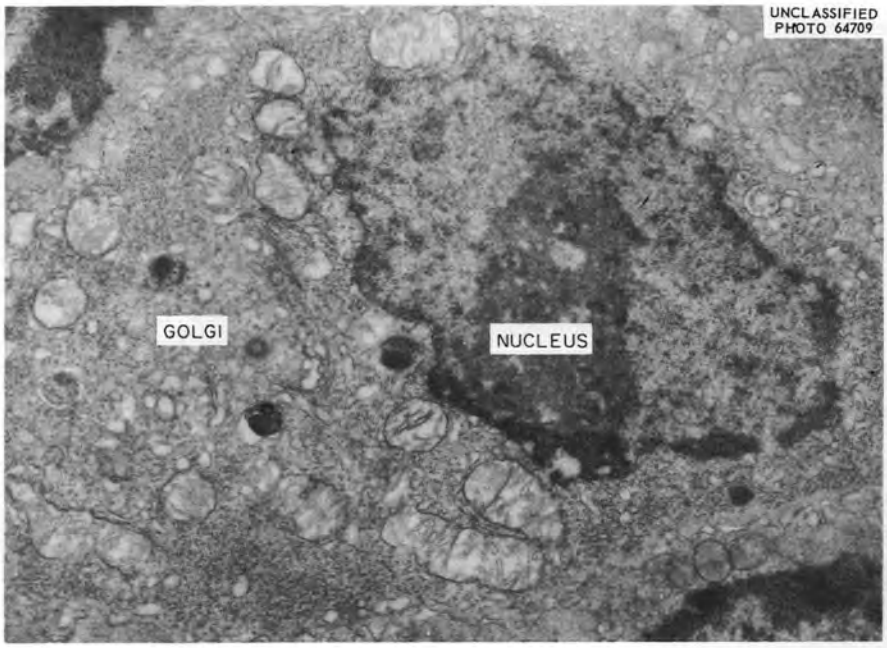

Fig. 10.5.2. Plasmablast Observed One Day After Antigen Injection. The Golgi region is well developed, a few profiles of endoplasmic reticulum (er) are present, and clusters of ribosomes characterize this stage. 15,000X. Reduced 29%.

cell is believed to be the mature cell in this family. On the origin of the plasma cell precursor, there is not good agreement. In this study, evidence from electron microscopic autoradiography is presented to show progressive stages of cytodifferentiation leading to the formation of plasma cells within a population of isotopically labeled cells found in spleen germinal centers.

Materials and Methods. – Adult (101 × C3H)F_1 mice received an intravenous injection of 2×10^9 sheep erythrocytes suspended in Tyrode's solution. One hour later each mouse received 100 μc of H^3-thymidine intravenously. At 4 and 24 hr after antigen injection, two mice were killed and spleens fixed for light and electron microscopy. Ultrathin sections mounted on copper grids were coated with a thin film of Ilford L-4 emulsion and allowed to expose for 70 days. The grids containing radioactive tissue were then developed, fixed, and stained in uranyl acetate.

Results and Discussion. – The labeled cells observed in germinal centers 4 hr after antigen administration are characterized by the abundance of free ribosomes in the cytoplasm (Fig. 10.5.1). These primitive cells are further characterized by their very sparsely developed endoplasmic reticulum. The pattern of cytodifferentiation observed within labeled germinal center cells is similar to embryonic development and differentiation and differentiation of regenerating tissue described in previous electron microscopic studies. As differentiation occurs in these cells, there is an apparent increase in the number of mitochondria. Also, the Golgi region develops from a few vesicles to an organized system of parallel membranes and vesicles. Helical arrangements of ribosomes are sometimes observed in the cytoplasm of these cells.

The most apparent difference in the ultrastructural organization of primitive germinal center cells and plasmablasts (Fig. 10.5.2), which are believed to be derived from the former cells, concerns the arrangement of ribosomes into clusters or rosettes. Thus, it appears that within this family of cells ribosomes are organized into clusters, perhaps polysomes, similar to the hemoglobin-synthesizing units described by Warner *et al.*[1] This occurs prior to the extensive development of endoplasmic reticulum, the outstanding ultrastructural feature of mature plasma cells. These results would seem to support the idea that antigenic stimulation induces the proliferation and differentiation of antibody-producing cells from a family of primitive or undifferentiated cells. It is also consistent with the idea that the immature plasma cell is an active protein-synthesizing cell prior to the formation of a mature plasma cell.

[1] J. R. Warner, A. Rich, and C. E. Hall, *Science* **138**, 1399 (1962).

10.6 A STUDY OF GERMINAL CENTER DISSOCIATION AND ITS RELATION TO THE PLASMA CELL REACTION OF THE PRIMARY IMMUNE RESPONSE

M. G. Hanna

Introduction. – Lymphatic tissue changes are a constant feature of the immune response in normal animals. Two consistent findings during early intervals of the spleen lymphatic tissue reaction to a primary antigenic stimulation are germinal center dissociation and the occurrence of large numbers of primitive lymphatic tissue cells.

The presence and general significance of primitive lymphatic tissue cells for reaction to antigenic material has been recognized for some time. Although germinal center dissociation has been previously described in lymphatic tissue, its relation to the plasma cell response still is not resolved. A view presented by Congdon and Goodman[1] conceives of germinal centers as sources of primitive lymphatic tissue cells, which, upon suitable stimulation, lose their architectural arrangement as a result of rapid proliferation and migration of cells through the associated lymphocyte mass and into the red pulp of the spleen and lymph node medulla where they transform into plasma cells.

The purpose of the present investigation was to provide more detailed information on the histologic changes in spleen white pulp germinal centers during the first four days of the primary immune response with emphasis on the first 24 hr. Histologic aspects of germinal center dissociation during these intervals after a primary sheep red blood cell stimulation were studied and correlated with the plasma cell response in the spleen red pulp.

Experimental Procedure. – The general plan of this study consisted of two main experiments: Experiment I was a histologic study of the spleen lymphatic tissue during the primary immune response with measurements of changes in germinal center number and volume. A concurrent study of the cellular changes in the peripheral blood was also made. Experiment II was an H^3-thymidine autoradiographic study of the cellular changes in spleen, as interpreted from imprint preparations, between days 1 and 4 of the primary immune response.

Results and Summary. – The results of these studies and their interpretation are:

1. An increase in mean germinal center volume (Fig. 10.6.1) and mean number (Fig. 10.6.2) of centers per longitudinal section occurs 2 hr after SRBC injection, followed by a rapid decrease in both parameters over the subsequent 22 hr. A recovery in both germinal center volume and number occurs between days 1 and 4 following injection. At day 4, a fourfold increase in germinal center volume was measured in the antigen-stimulated group. Based on the histologic studies, following an initial proliferation germinal center dissociation is a loss of cells from the center by migration, resulting in centers of decreased volume.

2. In the SRBC-stimulated animals the increase in number of primitive lymphatic tissue cells in the lymphocyte mass coincides closely with germinal center dissociation or decreased germinal center volume. Topographically, the time sequence of the increase in number of primitive lymphatic tissue cells is first in the area of the dissociated germinal center, second in the edema-like region of the lymphatic nodule, and third in the red pulp of the spleen.

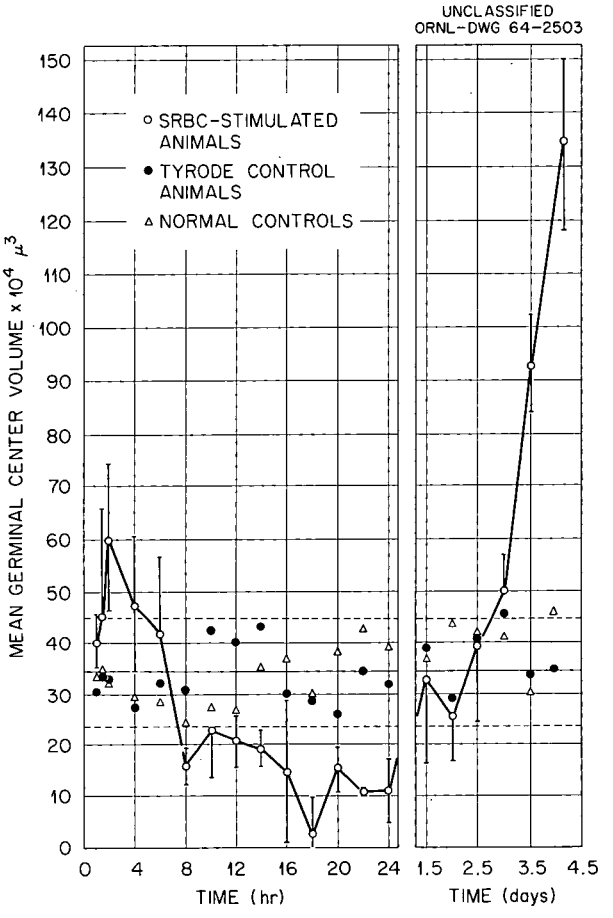

Fig. 10.6.1. Mean Germinal Center Volume. All means based on observations on five animals except at the 2-hr interval, where each mean is based on observations on ten animals. Perpendicular bars represent one standard deviation from the mean.

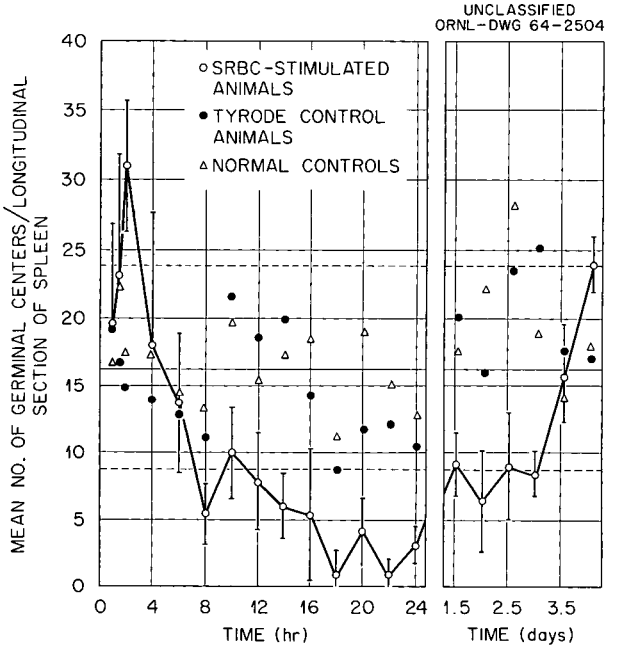

Fig. 10.6.2. Mean Number of Germinal Centers per Longitudinal Section of Spleen. All means based on observations on five animals except at the 2-hr interval, where each mean is based on observations on ten animals. Perpendicular bars represent one standard deviation from the mean.

3. An early lymphocytosis in the peripheral blood of the SRBC-stimulated animals 1 hr after injection coincides with a marked depletion of small and medium lymphocytes in the spleen red pulp resulting from RBC congestion in the red pulp region.

4. Germinal center cells respond to antigenic stimulation by proliferation which can be detected in two ways: (1) within intact germinal centers (hyperplasia), and (2) in a dispersed manner with migration. Both of these responses are considered characteristic of the inductive phase of the primary immune response, resulting in a local accumulation of large numbers of primitive lymphatic tissue cells in the spleen red pulp.

5. Coinciding with the increased number of primitive lymphatic tissue cells in the spleen red pulp was the formation of foci of plasma cells in which active plasmacytopoiesis could be observed.

The fate of primitive lymphatic tissue cells during early intervals of the primary immune response was interpreted from spleen imprint autoradiographs. These studies suggest that the primitive lymphatic tissue cells of the dissociated germinal centers are precursors of immature plasma cells, the transformation beginning between days 1 and 2 after antigen injection (Fig. 10.6.2).

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

10.7 LONG-TERM PRESERVATION OF MOUSE BONE MARROW STORED IN LIQUID NITROGEN

L. H. Smith Phan The Tran

Introduction. – Reproductive capacity of isologous mouse bone marrow after preservation at low temperature can be measured by the ability of the cells to promote recovery of lethally irradiated mice. Results of previous tests[1] of mouse marrow (frozen in Tyrode's solution containing 15% glycerol) made after storage at −30, −70, or −196°C for various times indicated that liquid-nitrogen temperature (−196°C) provided the best long-term storage (Table 10.7.1, Sect. A). Superiority of −196°C over −70°C was suggested on the basis that the cell loss and cell clumping observed after 22 weeks at −70°C was absent in the −196°C preparation. At the time these tests were made, batches of marrow frozen in Tyrode's solution containing 15% glycerol were set aside at −196°C for further testing.

Methods. – Although for irradiated recipients it would have been desirable to use the same type hybrid as used in the first experiments, namely, $(C57BL \times 101)F_1$, these were not readily available. Instead, the $(C3H \times C57BL)F_1$ was employed. Otherwise the procedures are the same as previously reported.[1]

Results and Conclusion. – It is apparent from results in Table 10.7.1, Sect. B, that bone marrow

Table 10.7.1. Long-Term Storage of Frozen Mouse Bone Marrow

X-ray exposure was 900 or 950 r; each mouse received 1.0×10^6 marrow cells intravenously in 0.5 ml[a]

	Number of Mice	Storage Temperature (°C)	Storage Time	Thirty-Day Survival (%)
A	25	−30	1 hr	83
	25	−30	4 weeks	57
	25	−30	25 weeks	0
	25	−70	1 hr	80
	25	−70	22 weeks	80
	25	−196	1 hr	68
	25	−196	4 weeks	80
	25	−196	25 weeks	72
	292			0 (x-ray control)
B	29	−196	29 months	79
	13			0 (x-ray control)
	16	−196	57 months	88
	10			0 (x-ray control)

[a]Section A data from M. A Bender, Phan The Tran, and L. H. Smith, *J. Appl. Physiol.* 15, 520 (1960).

cells retained their reproductive capacity for almost 5 yr at −196°C. Upon thawing, this preparation contained a slight amount of white fibrous material. The cell count was also reduced, which, in part, probably resulted from a moderate degree of clumping observed microscopically. The eosin uptake was 58%. Further biennial testing of the stored marrow is anticipated.

[1]M. A Bender, Phan The Tran, and L. H. Smith, *J. Appl. Physiol.* **15**, 520 (1960).

10.8 SHORT-TERM CULTURE CONDITIONS FOR MOUSE BONE MARROW CELLS

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

Introduction. – A study has continued of short-term (6-hr) culture conditions that will allow evaluation of in vitro postirradiation treatment of mouse bone marrow cells.[1] This report describes effects of several culture conditions for normal marrow cells.

Methods. – Reproductive capacity of suspended marrow cells was assessed by measuring their erythropoietic potential in lethally x-irradiated mice. This method, which involves spleen uptake of Fe^{59}, has been described previously.[2] Maintenance of erythropoietic capacity for 6 hr at 37°C was determined by comparing time zero values (fresh marrow) with those obtained after 6 hr culture. Cells were incubated in stoppered 25.0-ml Erlenmeyer flasks. Culture volume was 8.0 ml, and cell concentration was 5.0×10^6 per ml.

Results and Conclusions. – A variety of incubation media was tested. None of those listed in Table 10.8.1 was superior to TC 199 + 25% calf serum. Using this medium, the effect of pH was studied. After pH adjustment of the suspension with CO_2, the suspensions were gassed with 5% CO_2 + air. It was possible to maintain a fairly uniform pH for 6 hr. There appeared to be a broad pH optimum of the marrow cells ranging from 6.6 to 7.8 (tested at intervals of 0.2 pH unit). It appears that the loss of erythropoietic activity during the 6-hr culture period is not directly related to lack of proper hydrogen ion concentration. Although the exact O_2 requirement for maintaining mouse marrow cells is not known, gassing the culture continuously with 95% O_2 + 5% CO_2 did not prevent loss of activity. It is possible that this gassing procedure provides inadequate O_2 tension, and that O_2 under pressure is required. Two cell concentrations other than the one usually used (5×10^6 per ml) were tested. At a concentration of 50×10^6 per ml the usual loss (~30%) was noted, whereas at 0.250×10^6 per ml the loss was about 75%. Whether or not there is an optimum cell concentration remains to be tested.

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

[2]L. H. Smith and T. W. McKinley, Jr., *Biol. Div. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 102.

Table 10.8.1. Effects of Several Media on Maintenance of Erythropoietic Capacity of Mouse Bone Marrow After 6 hr Incubation at 37°C

Medium	$\frac{\text{Time Zero Value}}{\text{6-hr Value}} \times 100$	Number of Experiments
Tyrode's solution + 10% calf serum	25	4
TC 199 + 1% calf serum	61	1
TC 199 + 10% calf serum	64	5
TC 199 + 25% calf serum	72	10
100% calf serum	31	1
TC 199 + 25% isologous serum	73	1
Whole isologous blood	73	1
TC 199	55	1

10.9 TEST FOR PARENTAL ANTIGENS NOT EXPRESSED IN F_1 HYBRID MICE

Joan W. Goodman

Introduction. – The possibility that an F_1 hybrid may fail to inherit all histocompatibility antigens present in both parents is of interest to those concerned with tissue transplantation. The presence of such antigens has been proposed on occasion to explain experimental results that appear to be inconsistent with the so-called law of tissue transplantation, based primarily on tumor acceptance or rejection in mice, that parental tissue is, in fact, completely acceptable to its F_1 hybrid.

Methods and Results. – Inbred mice (P1) received at birth an injection of spleen cells from an F_1 hybrid cross between that strain (P1) and a second inbred strain (P2). When these mice were 10–20 weeks old, they were grafted with F_1 skin. Parental strains used in these experiments were C57BL, C3H, C57BL/6, and DBA/2; F_1 hybrids were $BC3F_1$ and $B6D2F_1$. P1 mice found to be tolerant (for at least 50 days) of F_1 were then grafted with P2 skin; all were tolerant of this as well. Neither F_1 nor P2 skin ever survived longer than 2 weeks on control P1 mice (those that received either isologous cells at birth or nothing at all). Some P1 mice that received F_1 cells at birth and that had not been previously grafted with F_1 skin, were found at 4–5 months of age to be highly tolerant (retaining grafts for 100 days) of P2 skin. None of these tolerant mice showed evidence of erythrocyte chimerism when tested just before or in the weeks following skin grafting. Furthermore, none of the tolerant P1 mice produced anti-P2 hemagglutinins, even when repeatedly immunized with P2 hemopoietic and lymphatic cells, whereas nontolerant control mice regularly did.

Conclusion. – In these experiments F_1 cells were able to induce tolerance of P2 antigens, and no evidence was obtained that antigens present in P2 cells were lacking from F_1 spleen. The possibility remains, of course, that such P2 antigens do exist but cannot be detected by the techniques employed.

10.10 FURTHER OBSERVATIONS ON MOUSE LEUKOCYTE CULTURE

Henrianne G. Willard

Introduction. – The present study is a continuation of work reported earlier.[1] Although short-term culture has been successfully applied to the leukocytes of humans, monkeys, oxen, snakes, chickens, hamsters, deer mice, rats, rabbits, and guinea pigs, no reproducible method has yet been developed for the mouse. Since the metabolic rate of most dividing cells is higher than that of nondividing cells, attempts were made to vary the culture conditions in order to vary the physiological state of the cultured cells.

Methods and Results. – Hybrid mice, (C3H/Anf Cum × C57BL/Cum)F_1, of both sexes were used in this study. Ages ranged from 2 to 20 weeks. Mice were narcotized with 1 : 10 Nembutal or with ether, and were bled by cardiac or tail puncture into a syringe or tube rinsed with heparin or EDTA (1% in 0.7% NaCl). Blood was used from individual mice, or was pooled in a chilled tube from a number of mice.

Leukocytes were generally separated by 3% dextran 1 : 1 (MW 275,000), or from the buffy coat obtained by centrifugation at 2–4°C at 1500 rpm for 15–20 min. Occasionally, heparinized whole blood was used for culture to assure the presence of all types of leukocytes.

Since many dividing cells require high oxygen concentrations, the gas phase above the cells was altered by bubbling oxygen into the culture vessel at various intervals during incubation: 1 hr at the beginning of culture, 6 hr at the beginning of culture, and intermittently throughout the culture period. Culture volume was also varied, since Nichols and Levan[2] had reported successful culture of mouse leukocytes by means of a semimicro method of culture. Cultures were maintained in 80-ml screw-cap prescription bottles, screw-cap tubes, 50-ml Erlenmeyer flasks, and 15- by 125-mm test tubes. These were placed at various angles in the incubator in order to vary culture depth. Suspensions were also cultured in darkness by means of black-taped bottles. Generally, a single variable was studied at a time.

Intact cells were present during the first few days of culture, with gradually increasing vacuolization and various indications of nuclear breakdown, including chromatin condensation, and disappearance of organized structure.

Conclusions. – If the leukocyte capable of mitosis is derived from lymphocytes, it might be expected that mouse blood would provide a greater number of dividing cells because of the high percentage of lymphocytes in the blood of this species. Three possibilities may be suggested here as reasons for the apparent inability of mouse leukocytes to grow in culture. First, the culture conditions may not be optimal for stimulation of mouse leukocytes. Second, the cellular division of leukocytes in other animals due to stimulation by phytohemagglutinin may have resulted from a previous encounter with the plant protein in nature, and may be an allergic response to the foreign protein. Third, the type of undifferentiated mononuclear cell still capable of division may not be present in the mouse. The latter possibility seems to be the most likely one at present.

[1]Immo Hoppe, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963,* ORNL-3427, pp. 74–75.

[2]W. W. Nichols and A. Levan, *Fundamental and Clinical Aspects of Radiation Protection and Recovery,* No. 4, pp. 40–41 (1961).

10.11 PARASITE SCREENING AND THE THERAPEUTIC VALUE OF ORGANIC PHOSPHATES IN INBRED MICE

M. L. Simmons H. E. Williams[1]
Everline B. Wright

Young adult mice of C57BL, C57BL/6, $1C3F_1$, $C31F_1$, C3H/Anf, 101, 5M, 21M, 14M, LP, LAF_1, BALB/c, CBA, C57L, CAF_1, DBA/2, BLF_1, $B6D2F_1$, $C3BF_1$, $BC3F_1$, $B1F_1$, and RF/Up strains used at the Biology Division of the Oak Ridge National Laboratory were examined for helminths, mites, and lice. Examination of 426 mice revealed infestation with the pinworm *Syphacia obvelata* was 53%, the cestode *Hymenolepsis nana* 13%, the mite *Myobia musculi* 13%, the mite *Mycoptes musculinus* 3.9%, and the louse *Polyplax serrata* 3.9%. The percentage infestation by the pinworm *S. obvelata* ranged from 0.0% in 7 strains to 100% in 8 strains; by the cestode *H. nana* from 0.0% in 14 strains to 100% in $C31F_1$; by the mite *M. musculi* from 0.0% in 19 strains to 100% in the RF/Up strain; by the mite *M. musculinus* from 0.0% in 21 strains to 100% in the RF/Up strain; and by the louse *P. serrata* from 0.0% in 21 strains to 100% in the RF/Up strain. The RF/Up mice were 12–15 months old, and all mice of the other strains were 6–12 weeks old. Ova of the parasites were recovered by the anal taping technique, zinc sulfate flotation, and sugar flotation. The adult worms were observed in diluted intestinal content, and the mites were observed after a detergent wash of the animal. A 14-day course of treatment with an organic phosphate cleared the mice of *S. obvelata* and *P. serrata,* and it reduced the incidence of *M. musculi* and *M. musculinus.*

[1]Pathology and Physiology Section.

11. Pathology and Physiology

Section Chief – A. C. Upton

Pathological Effects of Neutrons and High-Energy Radiation in Mammals (11.1–11.4)

G. E. Cosgrove[a]
E. B. Darden, Jr.[a]
W. H. Benedict[b]
K. W. Christenberry[b]
J. W. Conklin
M. L. Davis[a]
V. K. Jenkins
Lois H. Jordan
H. E. Williams

Gnotobiotics (11.9, 11.11)

H. E. Walburg, Jr.
L. C. Gipson
Edna I. Mynatt
Lillie B. Osborne
G. D. Price
D. M. Robie
F. J. Stenglein, Jr.
B. L. Wood

Physiological and Chemical Effects of Radiation (11.5)

T. T. Odell, Jr.
L. J. Serrano
Carlene C. Amsbury
W. B. Anthony
J. F. Hacker III
C. W. Jackson
T. P. McDonald

Virology (11.3, 11.10)

R. L. Tyndall
Ernestine Teeter
J. G. Vidrine

Epidemiology and Microbiology

R. C. Allen
J. H. Chandler, Jr.
Jacqueline C. Holloway
R. T. Jones
Dorothy J. Moore

Immunopathology and Immunogenetics (11.6–11.8)

Gustavo Cudkowicz
Michael Bennett
L. C. Satterfield
G. M. Shearer

Histology and Autoradiography (11.10)

W. D. Gude
Mary S. Gude
D. A. Jones
Tennyson Mack
Rebecca G. Phillips
B. Kay Trosko

[a]Dual assignment.
[b]Consultant.

Cooperative NIH-AEC Chemical Co-Carcinogenesis Program (11.1, 11.2, 11.4, and 11.11)

11.1 LATE SOMATIC EFFECTS IN MICE TREATED WITH 4-(*p*-DIMETHYLAMINOSTYRYL)QUINOLINE[1]

G. E. Cosgrove M. L. Davis
J. W. Conklin

Studies[2,3] on radiomimetic chemicals disclosed that 4-(*p*-dimethylaminostyryl)quinoline (4M20)[4] had pronounced early toxic effects on the liver. Residual liver cell changes indicated that carcinogenic delayed effects might occur. Survivors of the early experiments and some additional new groups were studied, and preliminary observations of the resulting data are reported here.

Materials and Methods. – Young adult female RF/Up or (101 × C3H)F_1 mice were used. Some

Table 11.1.1. Some Delayed Somatic Effects of 4M20 in Mice

	Mouse Strain			
	RF		(101 × C3H)F_1	
	Controls	4M20	Controls	4M20
Number of animals	99	58	192	31
Incidence of liver lesions, %				
All types	4	88	10	90
Nodularity	0	74	6	71
Hepatoma	0	21	5	23
Mean age at death (days)	537	465	866	683

of the mice of each strain received 4 mg of 4M20 dissolved in 0.2 ml of distilled water (final pH 1.4) intravenously, which was approximately an LD_{50} dose. One group of RF mice served as injection controls, receiving an intravenous injection of acidulated water at pH 1.4. Nontreated control mice of the same strains were set up concurrently. The mice were observed throughout life, and each mouse was autopsied after death.

Results and Discussion. – In the agouti brown mice of the (101 × C3H)F_1 strain, mild graying of the fur was noted after injection of 4M20. Cataract formation was also caused by 4M20, as reported elsewhere.[5] Marked gross and histological abnormalities of the liver were noted in mice of both strains which had received 4M20 (Table 11.1.1). These abnormalities included scarring and nodularity, degenerative fatty infiltration, and hepatomas. The incidence of other neoplasms did not appear to be influenced. In both strains of mice, some life shortening resulted from 4M20.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]M. Asano *et al.*, *Arch. Pathol.* **75**, 250 (1963).

[3]We are indebted to Dr. Carl Bahner, Carson-Newman College, Jefferson City, Tenn., for supplies of 4M20.

[4]M. L. Davis *et al.*, *Lab. Invest.* **12**, 991 (1963).

[5]K. W. Christenberry *et al.*, *Arch. Opth.* **70**, 250 (1963).

11.2 EFFECT OF RADIATION DOSE, PARTIAL SHIELDING, AND RADIOPROTECTIVE CHEMICAL (AET) ON THE INDUCTION OF RADIATION GLOMERULOSCLEROSIS IN MICE[1]

G. E. Cosgrove M. L. Davis
L. H. Smith[2]

Previous studies have disclosed a high incidence of glomerulosclerosis in heavily irradiated mice. The present experiment was designed to study the influence of radiation dose, partial body shielding, and a radioprotective chemical (AET) on glomerulosclerosis and other late effects in mice.

Methods and Results. – The mice were ten-week-old males and females of the LAF_1 strain. They were administered 500 to 1200 r of 250-kvp x rays to the whole body, to the lumbar area alone (remainder of body shielded), or to all the body except the lumbar area (lumbar area shielded). Some groups of lumbar-exposed mice received 8 mg of AET per mouse 15 min prior to irradiation. Control groups were treated similarly except that no radiation exposure was given. Survivors of all groups were observed until death and then necropsied. The incidence of glomerulosclerosis was negligible in controls of all ages. At about 1 yr of age (~9 months postirradiation), animals that had received an exposure of 650 r or greater to the kidney began to develop glomerulosclerosis, the final incidence in each group increasing with the radiation exposure (Table 11.2.1). Exposure

Table 11.2.1. Incidence of Glomerulosclerosis as Influenced by X-Ray Dose, Partial Body Shielding, and AET

Radiation Exposure (r)	Incidence of Glomerulosclerosis (%)			
	Whole Body Irradiated	All but Lumbar Area Irradiated	Lumber Area Alone Irradiated	
			Without AET	With AET
500	7		8	
600			13	
650	31	0	31	
800			70	67
850			81	
1000			83	82
1200			92	76

of the kidneys was necessary for the induction of the disease. Pretreatment of some exposure groups with AET did not result in a significant reduction in subsequent glomerulosclerosis (Table 11.2.1).

Discussion. – The LaF_1 mouse has a lower incidence of spontaneous glomerulosclerosis than some other strains of mice, in which it appears consistently with aging. In the irradiated LAF_1, it appears to be predominantly radiation-induced. It was hoped that various radioprotective and recovery treatments would be as effective in reducing this delayed effect of radiation as they are in reducing the acute effects of radiation. In this experiment, the two such treatments used had different effects on the kidney lesion studied. Shielding of the kidney area prevented the development of glomerulosclerosis, whereas pretreatment with AET was largely ineffective. Our results with shielding confirm earlier observations, indicating that the pathogenesis of this disease depends on direct effects of radiation on the kidney. The results with AET require further analysis before their full significance can be evaluated.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]Mammalian Recovery Group.

11.3 TAILED PARTICLES ASSOCIATED WITH MYELOID LEUKEMIA IN RF MICE

W. W. Harris[1] V. K. Jenkins R. L. Tyndall
N. G. Anderson[2] C. L. Burger[2]

Introduction. – Attempts to identify and isolate the virus responsible for transmission of radiation-induced myeloid leukemia in RF mice have been carried out with the ultracentrifuge and electron microscope. These efforts have disclosed tailed particles morphologically similar in many respects to the Rauscher and Moloney murine leukemia viruses.

Methods and Results. – Cell-free ultracentrifugates of spleen brei and blood plasma from RF mice with passaged radiogenic myeloid leukemia have been found consistently to transmit the leukemia to unirradiated isologous recipients and to contain tailed particles of viral morphology (Fig. 11.3.1), as well as the spherical virus-like particles described earlier in cells.[3] In negatively stained preparations, the newly found particles are characterized by hexagonal beads 85 to 100 mμ in diameter with taillike projections up to 100 mμ long and 12 to 20 mμ wide. A possibly granular internal structure appears to be present in the heads of the large particles. Occasional particles show two tails, as has been observed with the Rauscher and Moloney viruses.

Discussion. – The presence in our leukemic mice of tailed particles, as well as spherical particles, is consistent with observations on mice rendered leukemic by the Moloney virus and by the Rauscher virus.[4] Whether the round and tailed particles are different viruses or merely different forms of the same virus in each case is not yet known. The presence of such particles in uninoculated mice developing leukemia after irradiation and their rarer occurrence in normal controls is compatible with the hypothesis that they represent a latent leukemogenic virus which is activated by irradiation. Investigation of this and other possible interpretations should be facilitated by identification and purification of the virus; hence, the detection and concentration of the particles described above may denote a significant step

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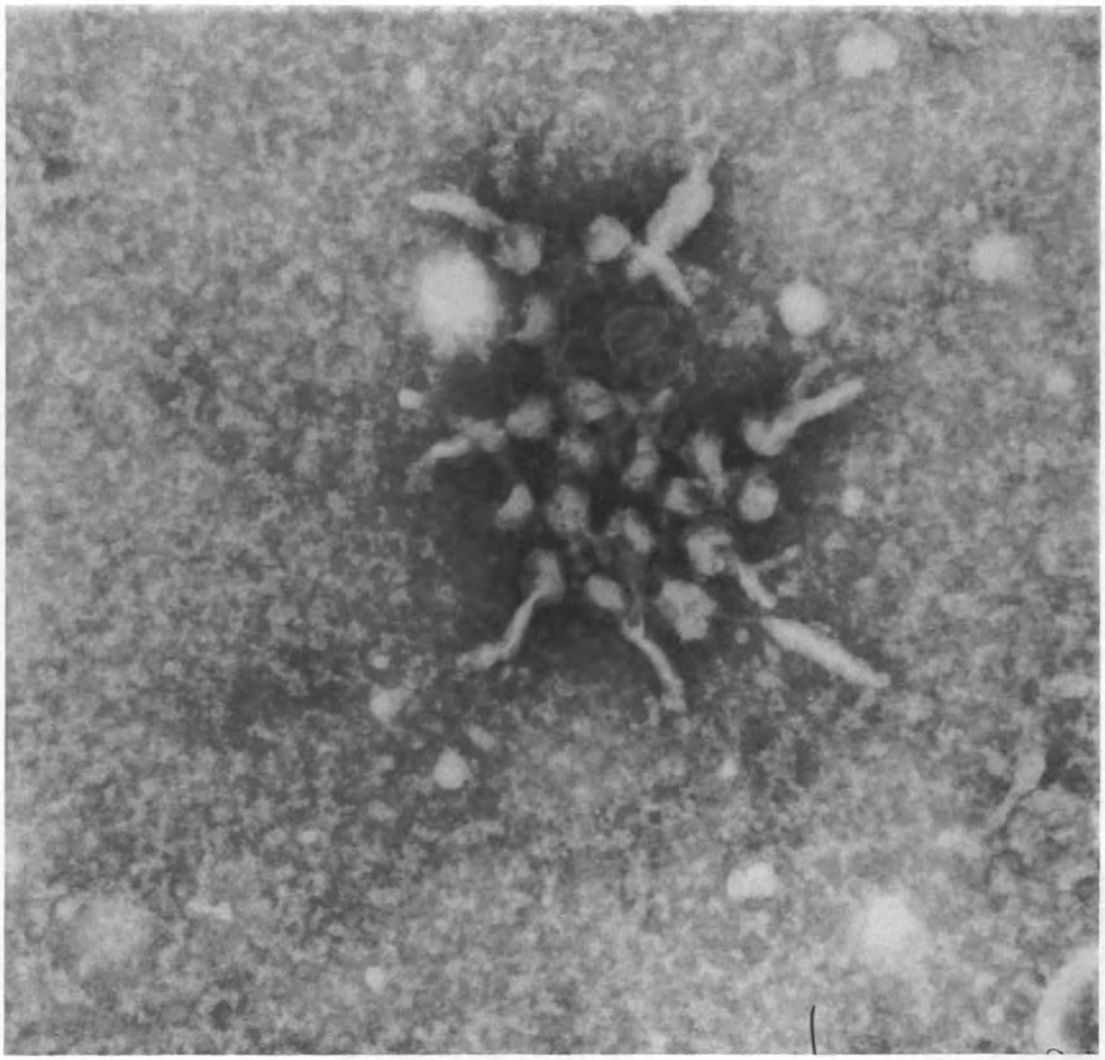

Fig. 11.3.1. Cluster of Virus-Like Tailed Particles from Pelleted Leukemic Plasma Ultracentrifugate. PTA negative stain. 60,000X.

forward in our study of the pathogenesis of the disease.

[1]Physics Department, Technical Division, ORGDP.

[2]Cell Physiology Group.

[3]D. F. Parsons *et al.*, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1962*, ORNL-3267, p. 94.

[4]J. B. Moloney, personal communication.

11.4 MOUSE POSTIRRADIATION LEUKEMIA: CONSISTENT OCCURRENCE OF AN EXTRA AND A MARKER CHROMOSOME[1]

Niel Wald[2] V. K. Jenkins
W. H. Borges[3]

Introduction. – The similarity between radiation-induced myeloid leukemia of the mouse and chronic granulocytic leukemia of man, and the regular

occurrence of the Ph^1 chromosome in patients with the latter disease, prompted a search for a similarly characteristic chromosomal abnormality in mice with this leukemia.

Results. – Post-x-irradiation granulocytic leukemia of the RF/Up mouse was serially transmitted by inoculation of RF/Up recipient mice with leukemic spleen cells, cell-free spleen ultracentrifugate, or plasma. All leukemic mice examined revealed a significant proportion of marrow cells with 41 chromosomes instead of the normal modal number of 40 and of marrow cells with a morphologically abnormal chromosome (Table 11.4.1). The morphological abnormality is an area of secondary constriction with achromatic staining properties on each chromatid, about two-thirds of the distance from the centromere to the distal end (Fig. 11.4.1); the two chromatids

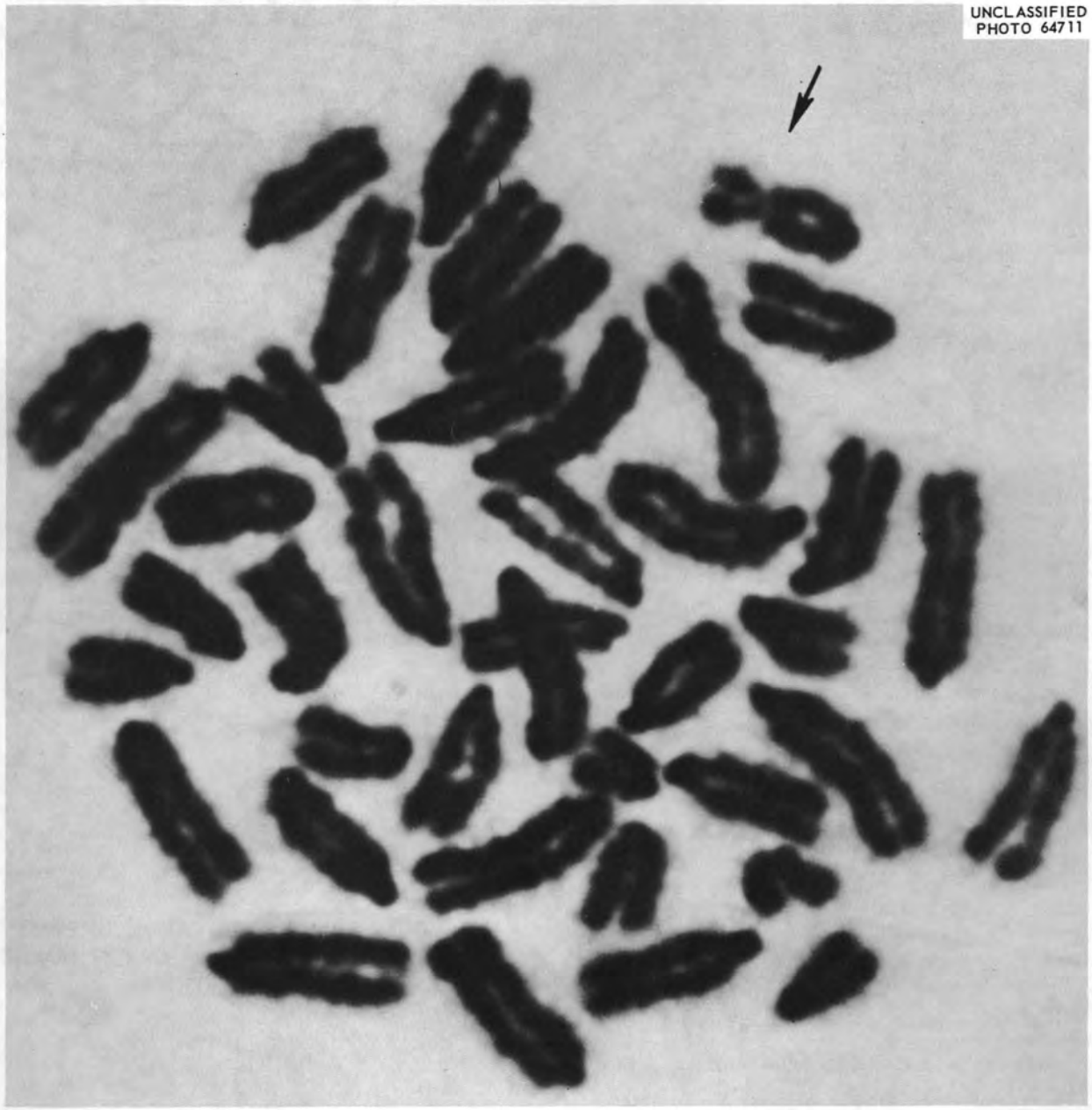

Fig. 11.4.1. Bone-Marrow Cell in Metaphase. Arrow indicates morphologically abnormal marker chromosome. Giemsa stain, light microscopy. 5000X. (Reduced 12.5%)

Table 11.4.1. Chromosome Counts and Marker Characteristics in the Bone Marrow Cells of RF/Up Mice Inoculated with Various Passage Materials from a Primary Post-X-Irradiation Granulocytic Leukemia

Number and State of Mice	PID[a]	Chromosome Counts							Marker Characteristics				
		<38	38	39	40	41	42	>42	Total with Marker	= 41 Chromosomes		≠ 41 Chromosomes	
										Marker	No Marker	Marker	No Marker
Leukemic Plasma, 21st Transfer Generation													
1 L[b]	35	1	1	1	5	17	1	1	6 (22)	6 (22)	11 (40)	0 (0)	10 (37)
Leukemic Spleen Extract, 22d Transfer Generation													
1 L	44	0	2	4	7	18	8	0	4 (10)	2 (5)	16 (41)	2 (5)	19 (49)
Leukemic Spleen Cells, 23d to 27th Transfer Generations													
1 L	9	1	1	1	10	6	1	0	5 (25)	4 (20)	2 (10)	1 (5)	13 (65)
1 L	13	0	1	0	7	12	0	0	12 (60)	9 (45)	3 (15)	3 (15)	5 (25)
1 L	13	1	2	0	6	9	1	0	c	c	c	c	c
1 L	13	0	0	0	1	9	0	0	5 (50)	5 (50)	4 (40)	0 (0)	1 (10)
1 L	31	0	1	1	7	11	0	0	13 (61)	9 (45)	2 (10)	4 (20)	5 (25)
4 N	d	3	0	6	44	1	0	0	0 (0)	0 (0)	1 (2)	0 (0)	53 (98)
Leukemic Spleen Extract, 23d to 27th Transfer Generations													
1 L	36	0	0	2	4	13	1	0	15 (75)	11 (55)	2 (10)	4 (20)	3 (15)
1 L	47	0	0	11	2	14	2	1	12 (60)	9 (45)	5 (25)	3 (15)	3 (15)
1 L[e]	48												
1 L	57	0	3	0	0	17	0	0	14 (70)	12 (60)	5 (25)	2 (10)	1 (5)
14 N	d	1	4	3	66	0	0	0	0 (0)	0 (0)	0 (0)	0 (0)	74 (100)
Noninjected													
24 N		4	4	15	239	10	0	1	0 (0)	0 (0)	10 (4)	0 (0)	263 (96)

[a]Abbreviations: PID, number of days post-injection after which mouse was killed; L, leukemic; N, normal. The number in parentheses below certain numbers indicates the percentage of total cells studied in that mouse which that number represents.

[b]Neonatal female recipient.

[c]Preparation unsatisfactory for detailed morphological study.

[d]Killed 133 to 140 days after inoculations.

[e]Died and decomposed before cytogenetic studies could be made.

almost invariably appear to adhere to each other in this region. This "marker" chromosome is consistently of medium size and not from one of the pairs identifiable by virtue of secondary constrictions adjacent to the centromere. It was seen in the female as well as in the males and does not resemble the Y chromosome. The marker was present in greatest frequency in cells having 41 chromosomes. Comparable abnormalities were not observed in the uninoculated controls or in the inoculated animals failing to develop leukemia during the period of observation.

Discussion. – The consistent occurrence of an extra and marker chromosome in the cytogenetically abnormal cells of the leukemic marrow is noteworthy. Since not all mitotic bone marrow cells sampled are necessarily leukemic, the absence of abnormality in some cells is not unexpected. The mechanisms of production of the extra chromosome and of the structural abnormality are unknown. In view, however, of the constancy and specificity of the observed changes, they appear different from the varied aneuploidization and structural abnormalities that have been noted during the growth of many other types of neoplastic cells.

That the chromosomal changes are induced by inocula presumed to be cell free is of particular interest. The transmissibility of the leukemia by cell-free filtrates containing virus particles has already been demonstrated. Tentatively, therefore, the leukemia and coexisting chromosomal abnormalities are virus-induced. Additional support for this assumption is provided by preliminary studies of another passaged leukemia from an independent primary post-neutron-irradiated granulocytic leukemic female RF/Up mouse, in which the same chromosomal changes appear.

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11.5 SURVIVAL STUDY OF RAT BLOOD PLATELETS BY DFP^{32} LABELING TECHNIQUE

T. T. Odell, Jr. T. P. McDonald

Introduction. – Survival studies of blood platelets have been carried out with a variety of isotopic labeling techniques. Diisopropylfluorophosphate labeled with P^{32} (DFP^{32}) is probably the platelet tag most commonly used in human subjects. Upon injection it is said to combine immediately and irreversibly with esterase enzymes in circulating platelets and other blood cells. The pool of DFP^{32} is believed to be available for only a short time after injection because of rapid combination and excretion; peak platelet activity occurs immediately after injection. Only diisopropylphosphate, a substance said to be chemically inert and rapidly excreted, has been found after cell destruction, so that reutilization of the label should not occur. The disappearance curves of platelets labeled with DFP^{32} have been interpreted either as being linear, indicating a loss of platelets by senescence, or exponential, thereby indicating random loss from the circulation. Actually, there are two questions involved, one concerning the manner of loss of platelets from the circulation and the other the validity of the assumptions concerning the DFP^{32} labeling technique.

In the experiments described here, two methods of studying platelet survival, both utilizing DFP^{32}, have been compared. Different conclusions might easily be drawn from examination of the results obtained by the two methods.

Single Injection of DFP^{32} (ref. 1). – Male Sprague-Dawley rats weighing about 300 g were injected intravenously (jugular vein) with 0.5 μg of DFP^{32} per gram of body weight. The specific activity of the DFP^{32} was 150 to 200 μc per milligram of of DFP. At intervals of 2 hr to 9 days after isotope injection, blood was collected from the abdominal aorta, diluted with saline (five parts saline to eight parts blood), and the platelets were separated by differential centrifugation. The platelets of each rat were washed twice with saline and resuspended in saline. The number of platelets, red blood cells, and white blood cells in each suspension was counted and samples were prepared for radioactivity counting in a gas-flow proportional counter.

Transfusion of Platelets Labeled in Vivo with DFP^{32}. – Sprague-Dawley rats weighing 400 to 500 g were injected intravenously (jugular vein) with 0.8 μg of DFP^{32} per gram of body weight. Two hours later their blood was collected from the abdominal aorta, diluted with saline, and the platelets were separated by differential centrifugation. The platelets were washed once with 4 ml of 1:1 mixture of saline and normal rat serum

and were resuspended in the same medium. The number of platelets and white blood cells in these suspensions was counted, and samples were prepared for radioactivity measurement.

One ml of these platelet suspensions containing 3.055 or 4.96 × 10^9 platelets was injected into the jugular vein of male Sprague-Dawley rats weighing about 300 g. Platelets were collected from these recipients one to four days later, and their radioactivity was determined.

Results and Discussion. – The points describing the radioactivity of platelets of rats given a single injection of DFP^{32} make a good fit to an exponential curve except at 2 hr, when the experimental points were above the curve, probably because of the high plasma activity at that time (Fig. 11.5.1). The half-time of platelet disappearance by this curve was about 2.9 days, the 10% level being reached about nine days after injection of DFP^{32}. The radioactivity of platelet-free plasma decayed with a half-time of about 26 hr.

The results obtained upon transfusion of DFP^{32}-labeled platelets conformed reasonably well to either a straight or an exponential line, and the 10% level was reached about 3½ to 4 days after platelet transfusion. An exponential curve drawn through these data indicated a half-time of platelet disappearance of about 24 hr in contrast with the 2.9-day half-time observed after single injection of DFP^{32}.

It is thus apparent that the disappearance curves of platelet radioactivity do not measure the same thing in the two kinds of experiments. It could be argued that the survival of transfused platelets was shortened because of damage during collection and transfusion. However, the maximum survival time observed in these transfusion experiments agreed fairly closely with times obtained by other methods and reported elsewhere, whereas the apparent survival time after a single injection of DFP^{32} was much longer. It presently seems likely that the curve of platelet radioactivity obtained after a single injection of DFP^{32} into rats does not actually measure platelet survival, but is extended by some function of the technique. Reutilization of label from defunct platelets would extend the curve. Another circumstance that would extend the curve is a release of labeled platelets over a period of a few days from labeled megakaryocytes.

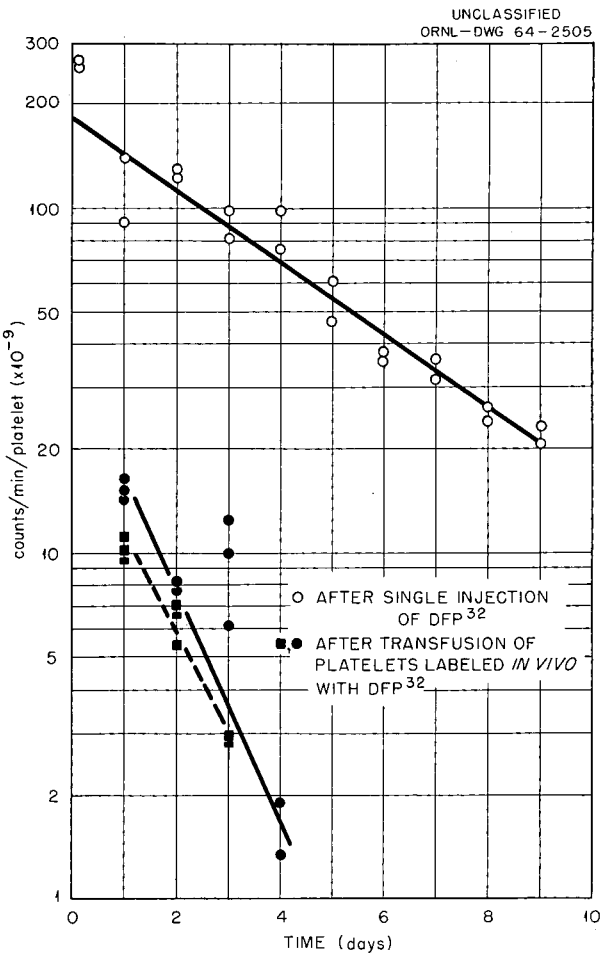

Fig. 11.5.1. Platelet Radioactivity After a Single Injection of DFP^{32} and After Transfusion of Platelets Labeled in Vivo with DFP^{32}. Each symbol represents one rat.

The method utilizing a single injection of DFP^{32} has provided estimates of the survival time of human platelets that agree reasonably well with estimates made by other means. The reason for the apparent difference between species remains to be explained.

[1] DFP^{32} supplied by New England Nuclear Corp.

11.6 LYMPHOCYTE CONTENT AND PROLIFERATIVE CAPACITY OF SERIALLY TRANSPLANTED MOUSE BONE MARROW

Gustavo Cudkowicz G. M. Shearer
L. C. Satterfield W. L. Hughes[1]

Introduction. – The ability of transplanted mouse bone marrow to colonize the spleen of an irradiated recipient is not dependent on the number of erythroblasts or myeloblasts inoculated, but rather on more primitive precursors.[2] Indirect evidence suggests that the precursor cell in question may be related to the small marrow lymphocyte. It was the purpose of this study to investigate (1) whether the failure of serially transplanted marrow to promote recovery of hemopoietic function in irradiated isogenic recipients[3] results from exhaustion of the marrow-lymphocyte pool, and (2) whether lymphocyte-deficient marrow can be restored by administration of isogenic lymph node cells.

Results. – (BALB/c × A/He)F_1 female mice were exposed to 850 r of x rays and received

Table 11.6.1. Effect of Serial Transplantation on the Ability of Marrow Cells to Promote I^{131}UdR Uptake in the Spleen of Irradiated Recipient Mice

Transplant Generation	Interval (days)		I^{131}UdR Uptake (%)[a]	
	Since Original Passage	Since Last Passage	Mean ± Standard Error of the Mean	Weighted Average and 95% Confidence Intervals
Controls				
(100–120 days old)			1.05 ± 0.05 (7)[b]	0.98
			0.97 ± 0.06 (9)	(0.90–1.06)
			0.88 ± 0.08 (5)	
(200–250 days old)			0.75 ± 0.05 (5)	0.81
			0.79 ± 0.09 (6)	(0.75–0.88)
			0.73 ± 0.07 (8)	
			0.97 ± 0.04 (9)	
First	30	30	0.59 ± 0.03 (5)	0.50
			0.52 ± 0.06 (5)	(0.45–0.55)
			0.44 ± 0.03 (10)	
	150	150	0.62 ± 0.05 (5)	0.55
			0.50 ± 0.07 (5)	(0.48–0.62)
			0.54 ± 0.03 (6)	
Second	65	30	0.29 ± 0.03 (7)	0.25
			0.22 ± 0.02 (8)	(0.21–0.29)
	150	115	0.35 ± 0.07 (2)	0.30
			0.23 ± 0.07 (3)	(0.23–0.36)
			0.31 ± 0.03 (7)	
Third	100	30	0.50 ± 0.05 (4)	0.43
			0.39 ± 0.02 (8)	(0.38–0.48)
	150	80	0.31 ± 0.02 (9)	0.38
			0.54 ± 0.01 (4)	(0.34–0.42)
Fourth				
(Without lymph node cells)	150	45	0.12 ± 0.04 (8)	0.08
			0.04 ± 0.007 (7)	(0.03–0.13)
(With lymph node cells)	150	45	0.19 ± 0.03 (5)	0.10
			0.13 ± 0.008 (9)	(0.08–0.12)
			0.03 ± 0.005 (9)	

[a]The uptake values for spleens of marrow-injected mice are given as percent of the total I^{131}UdR radioactivity administered *above* percent retention in spleens of uninjected radiation control animals.

[b]Each value denotes results for a single donor (number of test-recipient mice indicated in parentheses).

Table 11.6.2. Effect of Serial Transplantation on the Relative Frequency of Different Cell Types in Marrow

	Controls		Transplant Generation							
			First		Second		Third		Fourth	
	100–120 Days Old	200–250 Days Old							Without Lymph Node Cells	With Lymph Node Cells
Interval (days)										
Since original passage			30	150	65	150	100	150	150	150
Since last passage			30	150	30	115	30	80	45	45
Number of cells counted[a]	600	600	1800	600	400	400	800	400	200	600
Frequency distribution of all types (%)										
Myeloblasts and promyelocytes	1	2	3	4	4	4	7	4	5	6
Myelocytes and metamyelocytes	12	14	14	15	17	18	24	29	17	24
Granulocytes	30	27	27	30	23	24	23	29	27	15
Erythroblasts	26	29	27	26	40	36	30	22	38	44
Monocytes and reticulum cells	5	2	4	3	7	7	5	3	4	4
Lymphocytes										
Large	2	2	2	3	1	1	1	2	2	0
Medium and small	21	22	18	17	2	7	8	9	4	2
Other cells[b]	3	2	5	2	6	3	2	4	3	5

[a]200 cells per donor mouse.

[b]This category includes megakaryocytes, mast cells, plasma cells, histiocytes, and cells damaged beyond identification.

5×10^5 isologous marrow cells. Marrow was retransplanted at 35-day intervals into secondary, tertiary, and quaternary recipients. The latter received in addition 10^7 fresh isologous lymph node cells. The proliferative capacity of the marrow was determined by estimating its ability to promote uptake of 5-iodo-2′-deoxyuridine-I^{131} (I^{131}UdR) into the DNA of recipient spleens and was correlated with its cellular constitution as revealed by differential cell counts. The proliferative capacity of the marrow appeared to be directly correlated with its lymphocyte content throughout the experiment (Tables 11.6.1 and 11.6.2). Both were decreased 150 days after the first transplant. With serial transplantation, the lymphocyte deficiency and the inability to proliferate were accentuated. Fresh lymph node cells did not correct these marrow deficiencies although they improved the survival of the recipients. Changes in erythroblast and myeloblast content did not appear to be correlated with the competence of the transplanted marrow for continued proliferation.

Conclusions. – The results reconfirm previous observations, suggesting the involvement of small, mononucleated, lymphocyte-like marrow cells in hemopoietic cell production. They also suggest that such a cell is not derived from lymph node lymphoblasts.

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[2]Gustavo Cudkowicz *et al.*, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 92.

[3]D. W. H. Barnes *et al.*, *Ann. N.Y. Acad. Sci.* **99**, 374 (1962).

11.7 PLURIPOTENTIAL STEM CELL FUNCTION OF THE MOUSE MARROW LYMPHOCYTE

Michael Bennett G. M. Shearer
Gustavo Cudkowicz

Introduction. – Studies on the morphology and function of regenerating bone marrow strongly suggest that the proliferative capacity and hemopoietic competence of the marrow depend on its content of small, lymphocyte-like mononucleated cells, which are capable of incorporating tritiated thymidine.[1,2] The structure and function of these "lymphocytes," however, as opposed to the lymph node and lymph lymphocytes, have not been thoroughly investigated, probably because of the complexity and heterogeneity of the cells among which they normally reside in the marrow and from which they have not been successfully isolated hitherto. This report describes an approach to isolate the marrow "lymphocyte" by removing other elements from suspended marrow cells through glass-wool filtration.

Results. – Marrow cells from long bones of normal adult or of polycythemic C3H and (C3H × C57BL)F_1 mice were suspended in Tyrode's solution at a concentration of 10^7 cells/ml. Aliquots of the cell suspension were filtered twice through columns of packed glass wool prepared according to the procedure of Hildemann *et al.*[3] The compactness of the columns was varied to obtain differing degrees of lymphocyte separation. Marrow samples were smeared on glass slides for microscopic examination and stained with Ralph's hemoglobin and Wright-Giemsa stains. It was possible by filtration to remove most of the large cells (i.e., blast forms, reticulum cells, histiocytes, and monocytes) and to retain small and medium lymphocytes, normoblasts with pyknotic nuclei, metamyelocytes, and granulocytes (Table 11.7.1). The extent of this lymphocyte "enrichment" was inversely related to the total cell yield and directly related to the compactness of the columns (Table 11.7.1, experiment 1). When polycythemic donors were used, their marrow was essentially devoid of identifiable erythroid precursor cells and its cellular composition on filtration further simplified (Table 11.7.1, experiments 3 and 4). Known amounts of the original and of the filtered marrow were transplanted into recipient mice exposed a few hours earlier to 900 r of x rays. The proliferative capacity of the transplanted marrow was estimated by measuring the marrow's ability to promote DNA synthesis in the spleens of the recipients, as judged by incorporation of 5-iodo-2′-deoxyuridine-I^{125} (I^{125}UdR), a specific DNA precursor, five or seven days after transplantation. The hemopoietic function of the transplanted marrow was estimated by examining histologic sections of the recipients, by recording their 30-day survival, and by determining whether F_1 mice given parental C3H marrow had donor-type hemoglobin in their peripheral red blood cells,

Table 11.7.1. Percentage of Different Cell Types in Mouse Marrow Before and After Glass-Wool Filtration[a]

Cell Type	Experiment 1, C3H Donors			Experiment 2, F_1 Hybrid Donors		Experiment 3, F_1 Hvbrid Polycythemic Donors		Experiment 4, C3H Polycythemic Donors	
	Unfiltered	Filtered (loose)[b]	Filtered (tight)[b]	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered
Number of cells counted	300	500	1000	2500	1500	500	500	3000	3000
Blast cells	0.7	0	0	1.0	0.9	0	0	0.7	0.2
Myeloblasts and promyelocytes	4.0	2.0	0	5.4	2.3	8.0	0.2	10.3	1.5
Myelocytes	2.0	1.4	0	3.3	0.7	3.6	0.2	5.0	0.4
Metamyelocytes	9.3	9.0	0.3	10.1	6.6	13.2	1.2	18.1	8.1
Granulocytes	33.7	42.4	34.9	28.4	25.7	52.8	57.8	41.5	48.4
Proerythroblasts and erythroblasts	21.7	3.2	0	13.3	2.6	0	0	0	0
Normoblasts	8.0	9.0	10.9	7.5	5.7	0	0	0	0
Histiocytes, monocytes, and reticulum cells	0.6	0	0	0.8	0.1	0	0	1.6	0.2
Lymphocytes									
Large	1.0	0	0	0.5	0.1	0.6	0	0.6	0.3
Medium	2.6	2.4	0.8	6.6	6.0	5.4	0.6	5.3	10.3
Small	16.3	30.6	53.1	23.1	49.1	16.6	40.0	17.0	30.5

[a]Cells damaged beyond identification and megakaryocytes were not counted. Intact cells of the latter type were not found in filtered marrow.
[b]In respect to the compactness of the glass-wool columns.

Table 11.7.2. Proliferative Capacity of Filtered and Unfiltered Mouse Bone Marrow in Relation to Various Cell Types[a]

Cell Type	Experiment 1,[b] C3H Donors			Experiment 2,[b] F_1 Hybrid Donors		Experiment 3,[b] F_1 Hybrid Polycythemic Donors		Experiment 4,[b] C3H Polycythemic Donors	
	Unfiltered	Filtered (loose)[c]	Filtered (tight)[c]	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered
All nucleated cells	0.07	0.12	0.18	0.15	0.27	0.15	0.27	0.92	1.66
	(0.06–0.08)	(0.09–0.16)	(0.16–0.21)	(0.11–0.20)	(0.25–0.28)	(0.11–0.20)	(0.21–0.35)	(0.64–1.32)	(1.38–2.02)
Lymphocytes, small and medium[d]	0.38 ± 0.04	0.37 ± 0.05	0.34 ± 0.02	0.51 ± 0.05	0.49 ± 0.01	0.68 ± 0.08	0.67 ± 0.07	4.12 ± 0.39	4.18 ± 0.21
Normoblasts[d]	0.89 ± 0.18	1.35 ± 0.24	1.69 ± 0.18	2.00 ± 0.24	4.70 ± 0.50				
Granulocytes[d]	0.21 ± 0.02	0.29 ± 0.03	0.53 ± 0.04	0.53 ± 0.05	1.04 ± 0.05	0.28 ± 0.03	0.47 ± 0.05	2.19 ± 0.21	3.48 ± 0.18

[a]Uptake values for spleens of marrow-injected mice are given as percent of the total I^{125}UdR radioactivity administered *above* percent retention in spleens of radiation control animals not injected with marrow; 95% confidence intervals are given in parentheses.

[b]I^{131}UdR uptake values five days after marrow grafting in experiments 1, 2, and 3; seven days after marrow grafting in experiment 4.

[c]In respect to the compactness of the glass-wool columns.

[d]Standard errors of the mean uptake values for each of the individual cell types were calculated from the data in Table 11.7.1, taking into account also the variance of the percentages of the individual cell types.

Table 11.7.3. Hemopoietic Repopulation of Lethally Irradiated (C3H × C57BL)F_1 Mice Injected with Normal and Lymphocyte-Enriched C3H Marrow

Number of Nucleated Donor Cells				Response of Recipients		
Cells of All Types		Lymphocytes Alone		30-Day Survival		Hemoglobin Type at 60 Days (Filtered)
Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	
$\times 10^5$	$\times 10^5$	$\times 10^5$	$\times 10^5$			
1.0	0.5	0.22	0.20	30/30	17/19	Donor[a]
2.0	1.0	0.44	0.41	28/30	18/19	Donor[a]

[a]100% of the recipients' hemoglobin was of donor type in all tested animals.

60 days after infusion of the filtered marrow.[4] Neither the filtration procedure nor the absence of blast cells was observed to reduce the proliferative and hemopoietic competence of the marrow, since I^{125}UdR incorporation (Table 11.7.2), hemopoietic colony formation in the spleens, 30-day survival (Table 11.7.3), and erythropoietic repopulation (Table 11.7.3) were accomplished by the filtered marrow. The uptake of I^{125}UdR in the recipient spleens appeared, furthermore, to be correlated with the content of small and medium lymphocytes in both the unfiltered and in the filtered marrows, and not with any other cell type (Table 11.7.2).

Discussion. – From these and earlier data,[1,2] it is reasonable to infer that the mouse marrow "lymphocyte" is capable of continued proliferation and endowed with pluripotential stem cell function. This and other properties[1,2] of the marrow "lymphocyte" set it apart from the lymph and lymph node lymphocyte.

[1]Gustavo Cudkowicz *et al.*, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 92.

[2]Gustavo Cudkowicz *et al.*, *Nature* **201**, 165 (1962); this report, Sect. 11.6.

[3]W. H. Hildemann *et al.*, pp. 236–63 in *Ciba Foundation Symposium on Transplantation*, Churchill, London, 1962.

[4]R. A. Popp and G. E. Cosgrove, *Proc. Soc. Exptl. Biol. Med.* **101**, 754 (1959).

11.8 HYBRID RESISTANCE TO PARENTAL MARROW GRAFTS: ASSOCIATION WITH THE K REGION OF THE H-2 LOCUS

Gustavo Cudkowicz J. H. Stimpfling[1]
L. C. Satterfield

Introduction. – F_1-hybrid recipient mice resist the growth of transplanted parental C57BL/10 marrow unless they are homozygous H-2^b.[2,3] It was the purpose of this study to investigate whether this resistance requires heterozygosity at the whole H-2 locus, which is longer than one crossover unit, or merely at any particular site therein.

Results. – "Recombinant" lines were used which carry H-2 alleles that resulted from intra-allelic crossing-over during the course of serial backcrossing of H-2^b/H-2^a heterozygotes to C57BL/10 partners.[4] One of the lines carried the D and M components of the H-2^a, and the other carried the K component of H-2^a, both on the C57BL/10 background. Mice of these "recombinant" lines, which are co-isogenic with the C57BL/10 strain, were mated with C57BL/10 females to produce F_1 hybrids. The hybrid mice were serotyped by hemoagglutination with reagents specific for components DMCK and H of H-2^a. Following exposure to 700 r of x rays, they were injected with 10^6 C57BL/10 marrow cells and five days later classified for resistance or susceptibility to the grafted cells, by measuring

Table 11.8.1. Growth of C57BL/10 Marrow in F_1 Hybrid Mice from Parental Lines Co-Isogenic for Regions of H-2

Recipients[a]	Heterozygosity for H-2 Components	Number of Mice	Mean Uptake of I^{131}UdR (%)[b] ± Standard Error of the Mean	Classification
(C57BL/10 × C57BL/10.A)F_1	DMCHK	11	0.03 ± 0.007	Resistant
(C57BL/10 × recombinant 1)F_1	DM	11	0.73 ± 0.08	Susceptible
(C57BL/10 × recombinant 2)F_1	K	10	0.02 ± 0.008	Resistant
C57BL/10 (control)	None	10	0.80 ± 0.05	Susceptible

[a]Female donors; recipients of both sexes.

[b]Uptake values for spleens of marrow-injected mice are given as percentage of the total I^{131}UdR radioactivity administered in excess of the percentage retained by spleens of radiation control animals not injected with marrow.

the incorporation of 5-iodo-2′-deoxyuridine-I^{131} (I^{131}UdR) into newly synthesized DNA in the recipient spleens.[3]

Hybrids that were heterozygous for all the tested H-2 components, that is, H-2^b/H-2^a, were resistant to C57BL marrow grafts (Table 11.8.1); heterozygotes for the K component only were also resistant and undistinguishable from the former hybrids in this respect. Heterozygotes for D and M, on the other hand, were susceptible and undistinguishable in this respect from homozygous isogenic recipients.

Discussion. – The findings extend earlier data on the histoincompatibility of H-2 heterozygous F_1 hybrids toward parental C57BL hemopoietic cell grafts. They indicate that the genetic factor controlling this trait is located in the K region of the H-2 locus.

[1]Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

[2]Gustavo Cudkowicz and J. H. Stimpfling, *Genetics* 48, 886 (1963).

[3]Gustavo Cudkowicz and Audrey Eisenstadt, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p. 108.

[4]J. H. Stimpfling and G. D. Snell, p. 37 in *International Symposium on Tissue Transplantation Santiago, Chile, 1961* (ed. by A. P. Cristoffanini and Gustavo Hoecker), Universidad de Chile, Santiago, 1962.

11.9 SURVIVAL OF X-IRRADIATED GERM-FREE MICE

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D. M. Robie G. E. Cosgrove

The first experiments on survival of irradiated germ-free mice, carried out at the Lobund Laboratory, University of Notre Dame, indicated little effect of the germ-free state on $LD_{50/30}$ values and dose-response curves[1] but prolongation of the survival time.[1,2] In 1962, a colony of germ-free mice was established in our laboratory, with the aid of the University of Notre Dame, which supplied us with breeding stock of the noninbred ICR strain. Irradiation studies on this colony have recently been begun, preliminary results of which are reported in the following.

Methods and Results. – To date, 163 conventional and 174 germ-free ICR mice have received single exposures of x irradiation from 600 to 1000 r administered in seven different experiments. The germ-free mice were housed in flexible film isolators and were examined routinely for bacteria, mycoplasma, parasites, and some viruses. The mice were irradiated within the germ-free environment while confined in tubes rotating on a turntable under the x-ray beam. Conventional mice were irradiated under identical conditions and were then removed from the isolator and returned to conventional surroundings.

From the results thus far (Table 11.9.1), the $LD_{50/30}$ values approximate 740 r for conventional

Table 11.9.1. Thirty-Day Survival of X-Irradiated ICR Mice

Dose (r)	Number Surviving/Number Irradiated			
	Male		Female	
	Conventional	Germ-Free	Conventional	Germ-Free
1050				
1000		0/5		2/7
950		4/12		5/14
900		15/23	0/7	6/22
850	0/17	11/16	0/9	5/7
800	4/18	13/17	2/17	13/14
750	6/18	13/15	6/10	7/7
700	11/17		10/17	8/8
650	8/8		10/10	
600	8/8		7/7	7/7

mice and 900 r for germ-free mice (Fig. 11.9.1), a difference of 160 r. It is apparent that the slopes of the dose-mortality curves are different for these two groups of mice. The mean survival time was lower in conventional mice at all levels of survival and failed to increase with increasing survival as in the gnotobiotic animals (Fig. 11.9.2), reported previously.[2]

In 22 conventional and 53 germ-free survivors of these experiments observed at least five months after irradiation for late somatic effects of radiation, two germ-free and two conventional mice have died with thymic neoplasia.

Discussion. – A significant difference in the $LD_{50/30}$ of germ-free and conventional mice might have been expected from results of experiments on the reduction of radiation-induced mortality by treatment with antibiotics.[3] The absence of such differences in $LD_{50/30}$ reported previously are unexplained.[1] From our results, large effects of microbial flora on the survival of x-irradiated mice are evident not only in the midlethal range but also in the range of low lethality. The physiopathologic basis for these differences in terms of mode of death is under investigation.

The development of thymic neoplasia in germ-free, irradiated mice is highly significant whether attributable to the presence of leukemogenic viruses in "germ-free" mice or to a nonviral etiology. The incidence and pathogenesis of

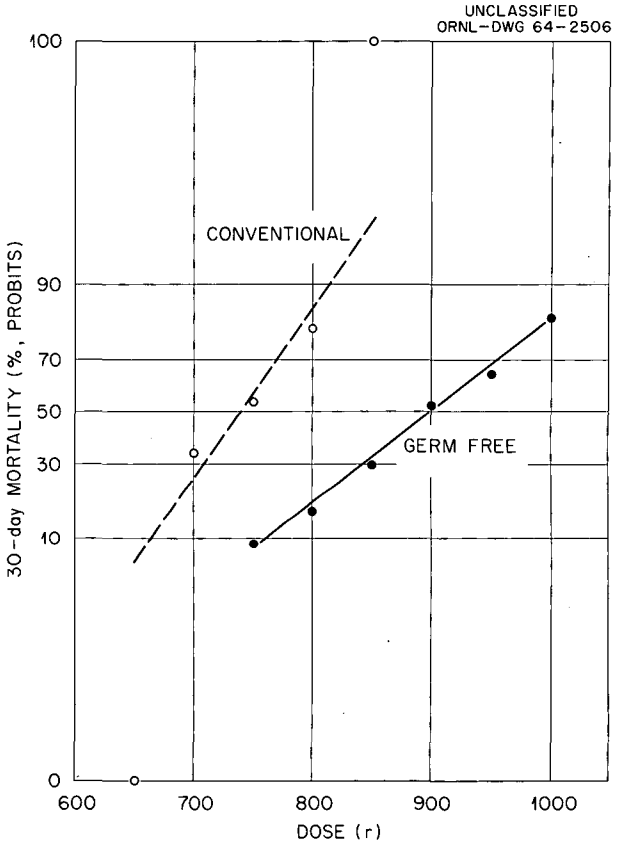

Fig. 11.9.1. Thirty-Day Mortality of Germ-Free and Conventional ICR Mice in Relation to X-Ray Exposure Level.

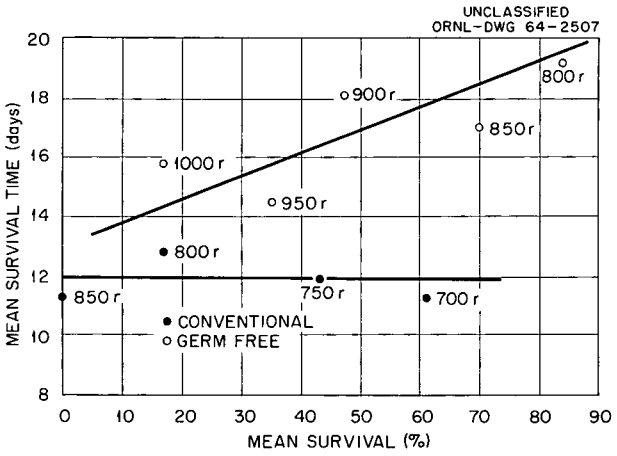

Fig. 11.9.2. Mean Time of Death in Germ-Free and Conventional ICR Mice in Relation to Percentage Dying Within 30 Days. The x-ray exposures, in roentgens, are indicated opposite each point.

these growths are subjects of a separate study being undertaken with virologically defined inbred RF/Up mice, as well as mice of several other low-leukemia and high-leukemia strains.

[1]B. R. Wilson, *Radiation Res.* **20**, 477 (1963).

[2]J. A. Reyniers *et al.*, *Radiation Res.* **5**, 591 (1956).

[3]C. P. Miller *et al.*, *Science* **111**, 719 (1950).

11.10 RESPONSE OF THE RF/Up MOUSE TO INFECTION WITH RAUSCHER LEUKEMIA VIRUS

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Introduction. – Recent studies have detected tailed virus particles in association with the virus-induced myeloid leukemia of the RF/Up mouse, which are similar in gross morphology to viruses causing lymphoid or reticulum cell leukemias in other mouse strains. Such similarity raised the possibility that the myeloid neoplasm in RF/Up mice reflected infection with one of these viruses rather than with a virus specific for myeloid leukemia. To test the reaction of the RF/Up mouse to infection with a lymphoid–reticulum-cell leukemia virus, three groups of RF mice were inoculated with varying doses of a 1×10^{-2} dilution of a highly leukemogenic pool of Rauscher virus.[1]

Methods and Results. – One group of newborn RF/Up mice was inoculated intraperitoneally with approximately 0.05 to 0.10 ml of Rauscher virus. A second group of ten-week-old male RF/Up mice was inoculated intravenously with ~0.25 ml of the same virus, and a third group of ten-week-old male RF/Up mice was inoculated intraperitoneally with approximately 0.5 ml of the virus. All the newborn recipients developed reticulum cell leukemia and died within 45 days after inoculation. Approximately 50% of the intravenously inoculated adult recipients developed similar leukemias and died within six months after inoculation. None of the intraperitoneally inoculated adult mice developed leukemia. Mice with leukemia showed intercapillary glomerulosclerosis of the type noted previously in association with transmitted radiogenic myeloid leukemia.

Discussion. – Since all leukemias produced were of the reticulum cell variety, it may be inferred that the virus causing myeloid leukemia in the RF/Up mouse is different from the morphologically similar Rauscher virus. It is also of interest that the glomerulosclerosis previously associated with the myeloid leukemia was present in all the reticulum cell leukemias induced with the Rauscher virus. It has also been noted in CF_1 mice rendered leukemic by the Rauscher virus. This lesion, then, is not specific for virus, neoplasm, or host strain. Its pathogenesis in relation to leukemia, however, remains to be determined.

[1]Kindly supplied by F. J. Rauscher, National Cancer Institute.

11.11 GENERATION CYCLE IN THE DUODENAL CRYPT CELLS OF GERM-FREE AND CONVENTIONAL MICE[1]

Sam Lesher[2] H. E. Walburg, Jr.
G. A. Sacher[2]

Introduction. – Cell division in proliferative tissues is known to be affected by a number of factors, such as starvation, hormone balance,

irradiation, and aging. Interference with cell renewal is, moreover, one of the major mechanisms in acute radiation injury. These experiments were concerned with the influence of the intestinal flora on the generation cycle of duodenal crypt cells and were carried out with germ-free and conventionally reared mice.

Methods and Results. – Noninbred ICR mice injected intraperitoneally with 10 μc of tritiated thymidine in 0.1 ml of sterile distilled water at 9:00 AM were killed at intervals from ½ to 26 hr thereafter, and segments of the duodenum were removed from them for autoradiographic and microscopic examination. The germ-free mice were maintained in a bacteria-free state throughout. All metaphase, anaphase, and telophase mitotic figures in ten sections were scored as labeled (more than five grains) or unlabeled. The duration of the generation cycle, consisting of four sequential stages: mitosis (M), presynthesis (G_1), synthesis (S), and predivision (G_2), was estimated by plotting the percentage of mitotic figures labeled against time after injection (Fig. 11.11.1). From this figure, the generation cycle time was estimated to be 13.6 hr for the duodenal crypt cells of the germ-free mice and 11.2 hr for the conventional mice (Table 11.11.1).

Table 11.11.1. Estimates of the Time Proliferative Cells of Duodenal Crypts of Germ-Free and Conventional Mice Spend in the Generation Cycle and Its Various Stages

Phase of Cycle	Time (hr)	
	Conventional Mice	Germ-Free Mice
G_1	3.0	3.4
S	6.5	8.0
$G_2 + M$	1.75	2.25
GC^a	11.2	13.6

[a]Total generation cycle.

Discussion. – The increased duration of the generation cycle in the germ-free mouse is due primarily to an increase in S and G_1. The data suggest, however, a general slowing of all phases of the generation cycle rather than of G_1 alone as had been suggested.[3] Our results confirm and amplify earlier observations indicating that the transit time of cells from the crypt to the villus tip is prolonged in the ileum of the germ-free mouse.[4] Whether the slower turnover of cells in the germ-free mouse results from decreased attrition of the cells on the villi or from nutritional deficiency, reduced metabolic activity, or other factors is under investigation.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill.

[3]H. Quastler, *Ann. N.Y. Acad. Sci.* **90**, 580 (1960).

[4]G. P. Abrams, H. Bauer, and H. Sprinz, *Lab. Invest.* **12**, 355 (1963).

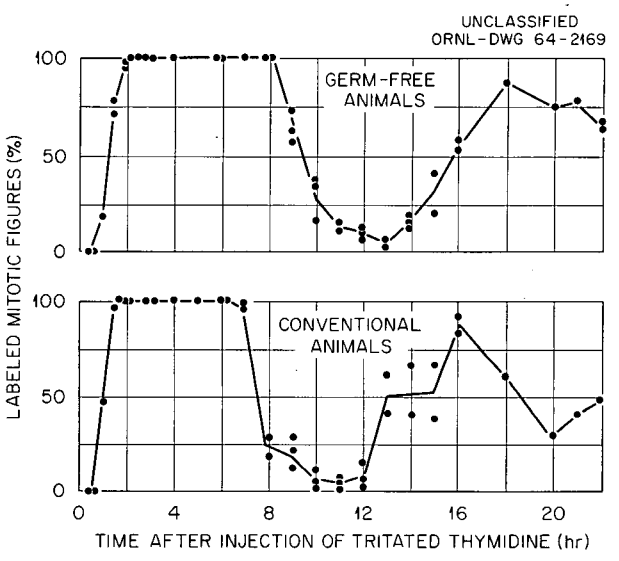

Fig. 11.11.1. Fraction of Mitoses Labeled vs Time Between Injection and Death. Each point represents one animal.

12. Chemical Co-Carcinogenesis

(Cooperative NIH-AEC Program)

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12.1 THYMINE DEFICIENCY AND "THYMINE-LESS" DEATH

A. P. Harrison, Jr. L. E. Franklin

Aerobacter aerogenes auxotrophs subject to "thymine-less" death have been isolated by the aminopterin–5-bromodeoxyuridine (AM-5BUdR) method.[1] One of these mutants, strain t23, will be described in detail. It will be compared with a similar *Escherichia coli* auxotroph, and also with the "classic" *E. coli* 15T$^-$.[2]

Strain t23 utilizes thymidylate; upon exhaustion of this compound death ensues, unless mass increase is prevented, as by glucose or oxygen exhaustion. The thymidylate requirement cannot be met by any of the following (added individually): thymidine triphosphate, deoxyadenosine-5′-monophosphate, deoxycytidine-5′-monophosphate, deoxyguanosine-5′-monophosphate, deoxyuridine-5′-monophosphate, deoxycytidine, deoxyuridine, adenosine, cytidine, guanosine, uridine, folic acid, or B_{12}. But the deficiency may be met with high concentrations of thymidine or thymine. Observe in Fig. 12.1.1 that these two compounds do not substitute for thymidylate on a simple mole-for-mole basis. This response is quite different from that of *E. coli* 15T$^-$ illustrated in Fig. 12.1.2, where thymine, thymidine, and thymidylate serve equally well as regards cell yield. (The growth rate of 15T$^-$ is slightly less in thymidylate, however.)

The above observations pose the following question: Does the AM-5BUdR method for acquiring thymine-less auxotrophs involve a different locus from that responsible for the thymine requirement in 15T$^-$? The answer is yes, because when *E. coli* strain 15 (the parental prototroph of 15T$^-$) is subjected to serial passage in AM-5BUdR, mutants like *A. aerogenes* t23 are obtained. One of these, strain 15-1, can be described with Fig. 12.1.1 by simply compressing the time axis twofold, since *E. coli* has about twice the generation time (Fig. 12.1.2).[3] An attempt to impose an additional thymine requirement by AM-5BUdR passage onto

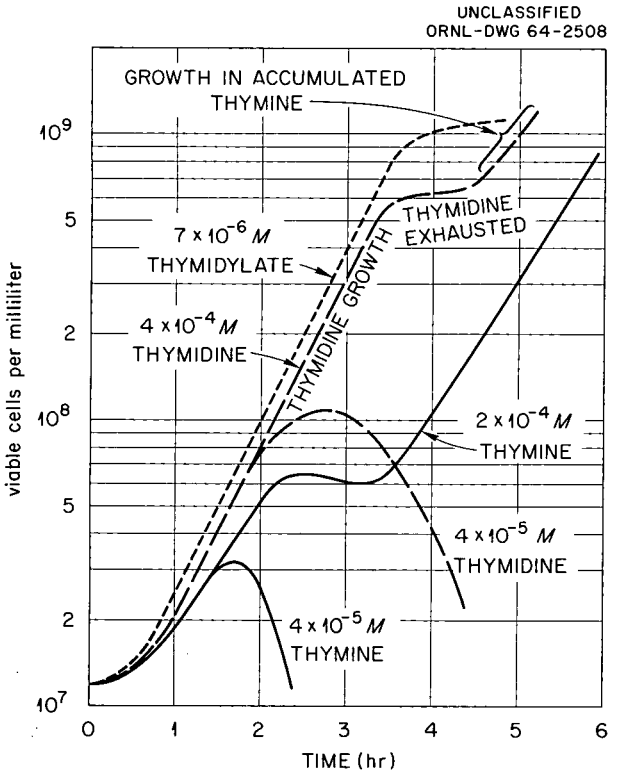

Fig. 12.1.1. Growth of *Aerobactor aerogenes* t23 in Glucose–Mineral Salts Medium with the Supplement Indicated. Glucose (0.3%) is yield-limiting at 2×10^9 cells/ml.

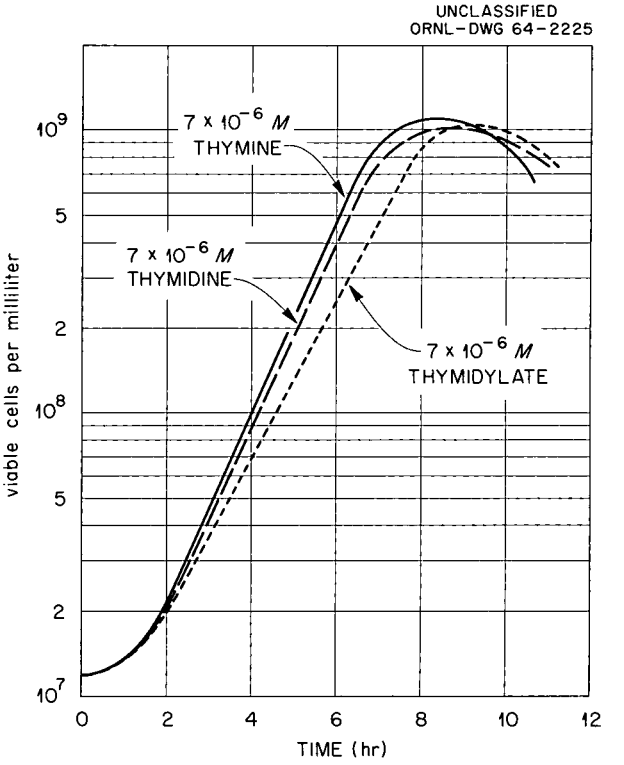

Fig. 12.1.2. Growth of *Escherichia coli* 15T⁻ in Glucose–Mineral-Salts Medium with the Supplement Indicated.

the already thymine-deficient 15T⁻ has been unsuccessful, possibly because of an unsatisfactory agar plate selection method.

The relatively low yield of t23 and 15-1 in thymidine is not due to a permeability defect – growth in low concentration of thymidine occurs at the maximum rate – but is due to breakdown of thymidine to thymine. Thus a t23 culture grown in 4×10^{-4} *M* (100 μg/ml) thymidine, after exhaustion of the thymidine, contains in its filtrate 2×10^{-4} *M* (25 μg/ml) thymine, and growth has two phases (Fig. 12.1.1), the second arising from subsequent utilization of the accumulated thymine. Below 4×10^{-5} *M* (10 μg/ml) thymidine, insufficient thymine accumulates to allow the second growth phase; cell decline begins within 30 min and continues unabated (Fig. 12.1.1). But between 4×10^{-5} *M* and 4×10^{-4} *M* thymidine, greater or lesser death will occur before the second growth phase begins. The presence of these compounds was detected by paper chromatography (water or water-saturated butanol) with subsequent water elution and photometric quantitation, and also by microbiological assay. Paper chromatograms from t23 and 15-1 culture filtrates (thymidine-grown cells) contain in addition to thymine a large amount of material with a strong blue fluorescence. Chromatograms from strain 15T⁻ contain neither thymine nor the fluorescent material. All chromatograms, however, have an ultraviolet-absorbing material in common, tentatively considered deoxyuridine.

The poor yield of t23 and 15-1 in thymine may be due to poor permeability to thymine, or to some regulatory mechanism holding the equilibrium in favor of thymine in reactions such as the following:

$$\text{thymine} + \text{deoxyuridine} \longleftrightarrow \text{thymidine} + \text{uridine},$$

$$\text{thymine} + \text{deoxyribosephosphate} \longleftrightarrow \text{thymidine} + \text{phosphate}.$$

Strain t23 in 4×10^{-5} *M* (5 μg/ml) thymine grows to a yield of 3×10^7 viable cells per ml; then thymine-less death ensues, although most of the

thymine remains available (Fig. 12.1.1). At 2×10^{-4} *M* (25 μg/ml) thymine and above growth is diphasic (Fig. 12.1.1). Two explanations (not mutually exclusive) are attractive: thymine may be utilized by two mechanisms and an ancillary reaction's products may affect the regulation of the thymine utilization reaction. (For example, the thymidine growth may be truly diauxic.) The following additional observations are pertinent:

1. Addition of 4×10^{-4} *M* deoxycytidine to a 4×10^{-5} *M* (5 μg/ml) thymine culture increases cell yield threefold.
2. This addition to a 2×10^{-4} *M* (25 μg/ml) thymine culture raises the first growth phase yield (shifts up the plateau) threefold.
3. Deoxyuridine is three times as effective as deoxycytidine in doing this.
4. The first growth phase can also be extended, but to a lesser degree, by reducing the phosphate concentration from 0.03 to 0.003 *M*.
5. Addition of cytidine to thymine cultures inhibits growth; deoxycytidine counteracts this inhibition.
6. Addition of deoxyuridine to thymidine cultures also improves cell yield.
7. Supplying thymidine in the form of thymidylate apparently prevents thymidine breakdown to thymine, since growth is smooth, not diphasic, and continues until exhaustion of the thymidylate.

The preferential and uncomplicated utilization of thymidylate by t23 poses an important question: Is thymidylate utilized *intact* for DNA synthesis? Unhappily, the answer is no, as the experiment described in the next paragraph attests. (Incorporation of thymidylate directly into DNA, for which there is no precedent, would provide an elegant system for nearest-neighbor analysis on DNA; for example, the thymine and phosphate moieties could be labeled simultaneously with C^{14} and P^{32}, respectively, providing convenient markers on the DNA strand.)

P^{32}-labeled thymidylate was prepared as follows. Wild-type *A. aerogenes* was cultivated in 400 ml of synthetic medium containing 0.003 *M* "cold" inorganic phosphate (with 0.05 *M* Tris buffer pH 7) and 12 mc of inorganic P^{32}. The entire culture was harvested at a cell density of 10^9 per ml. (One-half the radioactivity was taken up by the cells.) The method of Marmur[4] was followed for the extraction of DNA using sodium lauryl sulfate for cell lysis but employing only one deproteinization before and after ribonuclease treatment. The P^{32}-labeled DNA was enzymatically hydrolyzed[5] and the nucleotides were separated by means of paper chromatography[5] using isobutyric-isobutyrate. One hundred fifty micrograms of thymidylate emitting 3×10^6 counts/min were obtained. For the experiment itself, 400 ml of synthetic medium inoculated with t23 and supplied with 9.2×10^{-2} μ*M* (30 μg) "hot" (6×10^5 counts/min) thymidylate, 3.0 μ*M* (980 μg) "cold" thymidylate, and 0.03 *M* inorganic phosphate buffer (pH 7) was employed. The culture was harvested upon thymidylate exhaustion, the cell density being 10^9 per ml. Unit volume of culture and culture filtrate demonstrated identical radioactivity (1.5×10^3 counts/min). Thus, not only is thymidylate dephosphorylated, but this phosphate joins that in the milieu, rather than being preferentially attached to some site at the cell surface. In the extracted DNA only 510 counts/min were detectable. (The yield of DNA was approximately 30%.)

Nevertheless, the thymidine (or thymine) of the thymidylate is utilized; and, apparently, it exists in a form (or location) rendering it unavailable for competing reactions. This investigation continues in order to discover the "block" responsible for the observed anomalies.

---

[1]T. Okada, K. Yanagisawa, and F. J. Ryan, *Z. Vererbungslehre* **92**, 403–12 (1961).

[2]Acquired from Seymour Cohen.

[3]One minor difference from t23: the thymidylate concentration for threshold response is somewhat higher.

[4]J. Marmur, *J. Mol. Biol.* **3**, 208–18 (1961).

[5]The authors are grateful for information concerning DNA hydrolysis from Elliot Volkin and for advice in chromatographic analysis from W. E. Cohn.

12.2 A NEW APPROACH TO A STUDY OF THE MUTAGENICITY OF POLYCYCLIC HYDROCARBONS IN *NEUROSPORA*

H. V. Malling

The polycyclic hydrocarbons are particularly interesting because they have been shown to be potent carcinogens in mammalian systems, but little is known about their mutagenicity. Some tests have been made on bacterial and fungal systems but the results of these tests show little

agreement. Many of these compounds are insoluble or slightly soluble in water, and the disagreement in the results of tests for mutagenicity on microbial systems may simply be due to a differential uptake of these compounds by the cells of different organisms. In forward and reverse mutation experiments with *Neurospora*[1] the potent carcinogens 20-methylcholanthrene and 1,2,5,6-dibenzanthracene exhibited no mutagenic effect. It is conceivable that no mutagenic effect was detected merely because these compounds did not enter the cells under the conditions of treatment which were used. This possibility has been investigated here.

Results. – Many of the polycyclic hydrocarbons are fluorescent, and the penetration and accumulation of these compounds inside the cell can be demonstrated readily by means of fluorescence microscopy.[2] *Neurospora* conidia, growing or not growing, were treated with 20-methylcholanthrene or 1,2,5,6-dibenzanthracene dissolved either in Tween-80 or in histamine. (The latter two compounds are used to increase the solubility of the hydrocarbons.) Examination of the conidia by fluorescence microscopy showed that neither of the polycyclic hydrocarbons was accumulated in the protoplasm of *Neurospora* conidia, even after long incubation. On the other hand, acridine orange, a structurally related fluorescent compound, was accumulated extensively giving the cytoplasm a bright red fluorescence and the nucleus a bright green fluorescence.

Conclusions. – One explanation for the failure to find a mutagenic effect of these potent carcinogens in *Neurospora* is that the conditions used did not permit the compounds to penetrate and be accumulated by the cells.

The application of fluorescence microscopy in the study of the mutagenicity of polycyclic hydrocarbons should provide a simple test for (1) the development of experimental conditions under which a particular interesting fluorescent carcinogen is known to penetrate the cell and (2) the selection of those fluorescent carcinogens that penetrate and are rapidly accumulated by *Neurospora* conidia under certain standard conditions.

[1]W. J. Burdette and D. H. Haddox, Jr., *Cancer Res.* **14**, 163 (1954).

[2]G. H. I. Sloane and C. N. Loeser, *Cancer Res.* **23**, 1555 (1963).

12.3 GROWTH RESPONSE TO 6-METHYLAMINOPURINE OF *NEUROSPORA* MUTANTS BLOCKED IN DIFFERENT STEPS OF THE ADENINE BIOSYNTHESIS

H. V. Malling

Introduction. – For tests of the mutagenicity of different compounds in *Neurospora*, it would be desirable to develop a semiquantitative forward-mutation method where certain biochemical mutants could be isolated from the wild type simply by plating the cells on a selective medium. Heslot[1] has developed such a system in *Schizosaccharomyces pombe*. He found that the purple mutants, blocked probably in the same step as the purple mutants in *Neurospora*, cannot utilize 6-methylaminopurine (MAP); double mutants, however, blocked in the purple step and in an earlier step in purine biosynthesis can utilize MAP and can therefore be selected from the purple mutant by plating the cells on minimal medium supplied with MAP. In order to develop a similar system in *Neurospora crassa* the growth response of different adenine mutants to MAP has been investigated.

Results and Discussion. – In Table 12.3.1 it can be seen that mutants which can utilize hypoxanthine to restore their adenine requirement also can use MAP and that neither *ad-8* nor *ad-4* mutants (adenine specific) can utilize hypoxanthine or MAP. It may therefore be assumed that MAP probably is converted to hypoxanthine.[2] These results with *Neurospora* mutants seem to be in contradiction to the results obtained in *S. pombe* where not even the purple mutants can utilize MAP. The two kinds of mutations in *Neurospora* which cannot utilize MAP are *ad-8* and *ad-4* mutants. If double mutants with a block in the *ad-8* or *ad-4* step and with prior blocks in purine biosynthesis can utilize MAP, then the same type of forward-mutation selection system can be developed in *Neurospora* as in *S. pombe*. The development of such a system would permit the investigation of the mutagenicity of various chemical carcinogens by a series of simple tests. The solution of this problem is especially important for experiments with compounds like the polycyclic hydrocarbons (including both carcinogens and noncarcinogens) that are relatively

Table 12.3.1. Growth Response of Adenine-Requiring Mutants Blocked at Different Steps in the Adenine Biosynthesis

Group Designation	Reaction Blocked	Mutants	Growth on Minimal Medium Supplied with		
			Adenine	Hypoxanthine	MAP
ad-4	AMPS ⟶ AMP	74A-Y112-M16	+	0	0
	SAICAR ⟶ AICAR	F-2	+	0	0
		F-12	+	0	0
ad-8	IM ⟶ AMPS	74-Y112-M343	+	0	0
		74A-K5-M12	+	0	0
ad-5	FAICAR ⟶ IMP	74-Y175-M253	+	+	+
	AICAR ⟶ FAICAR				
ad-1		74A-Y234-M394	+	+	+
		74A-Y234-M419	+	+	+
ad-3A		05-0006-0001	+	+	+
	AIR ⟶ (CAIR)				
	⟶ SAICAR				
ad-3B		05-0006-0024	+	+	+
ad-2	FGAM ⟶ AIR	1-0175-0256	+	+	+
ad-6	FGAR ⟶ FGAM	1-0175-0221	+	+	+
ad-9	GAR ⟶ FGAR	74A-Y154-M37	+	+	+
ad-7	Unknown but before *ad-3*	1-0175-0265	+	+	+

insoluble in water. Tests under a variety of conditions may be required to classify such compounds in terms of their relative mutagenicity.

[1]Reported orally at the Genetics Congress in The Hague, 1963.

[2]D. E. Duggan *et al.*, *Biochim. Biophys. Acta* **68**, 519–25 (1963).

12.4 BONE MARROW TRANSPLANTATION AFTER INTRAPERITONEAL ADMINISTRATION OF DMBA

C. C Congdon[1] D. G. Doherty[2]
Frances E. Hacker[1]

Introduction. – The polycyclic hydrocarbon 9,10-dimethyl-1,2-dibenzanthracene (DMBA) is a well-known chemical carcinogen in mice. In previous reports, attempts to reverse the acute

chemical toxicity of DMBA by AET protection and bone marrow transplantation were described.[3] These experiments are part of a program aimed at investigating the acute pathologic effects of representative members of the major classes of chemical carcinogens and certain pathogenic viruses in order to carry out protection and recovery experiments that might alter the pathologic changes.

In the earlier report,[4] AET protection and bone marrow transplantation did not prevent the mortality resulting from lethal doses of DMBA, although in one instance marrow injection was associated with fast recovery of the damaged hemopoietic system.

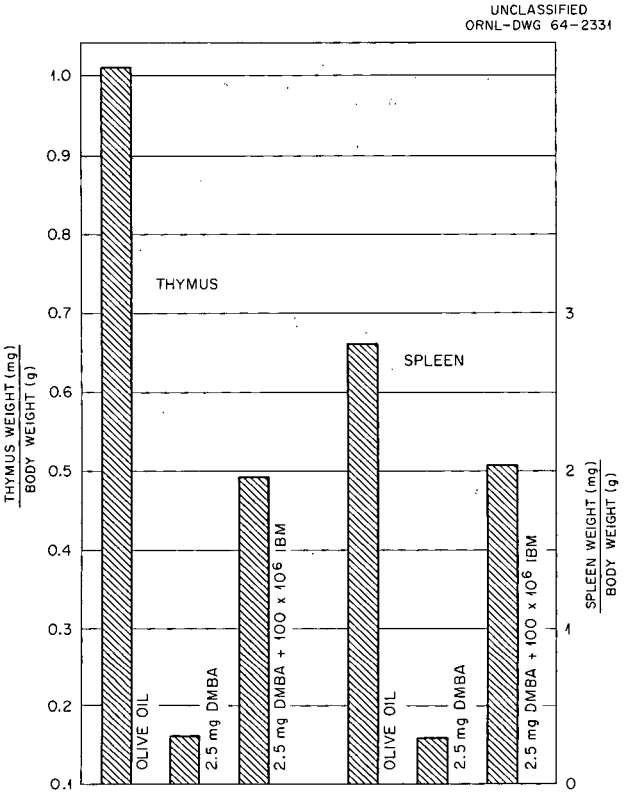

Fig. 12.4.1. Spleen and Thymus Body Weight Ratio 14 Days After DMBA in a Successful Experiment.

The present experiments showed in a clear fashion that marrow injection aided hemopoietic recovery from the toxic effects of intraperitoneally administered DMBA even though it did not increase 30-day survival.

Methods and Results. – Groups of $B6D2F_1$ male or female mice were given intraperitoneally 1.5 or 2.5 mg of DMBA dissolved in olive oil. Three days later half of the animals received 100×10^6 or, in one instance, 40×10^6 isologous bone marrow (IBM) cells intravenously. On the 7th and 14th day after DMBA, mice were killed and autopsied from the control and the bone-marrow-treated group. Animals that survived to days 21 and 28 were also examined in some experiments. Body, spleen, and thymus weight were taken at autopsy, and many tissues removed for histologic study.

In three experiments, there was greater histologic recovery of the spleen, bone marrow, thymus, and lymphatic tissues in the marrow-treated group than in the DMBA control. In a fourth experiment, there was no difference. Figure 12.4.1 shows the spleen and thymus body weight ratio 14 days after DMBA in one of the successful experiments.

Isologous bone marrow did not increase survival of mice after intraperitoneal DMBA because of the peritonitis produced when this route of administration was used. Pancreatic necrosis was present with the peritonitis. Death from peritonitis rather than hemopoietic failure was the major autopsy finding in all of the DMBA experiments.

Discussion. – Further experiments are now being carried out with the oral route of administration of DMBA to try and achieve a hemopoietic death that might be prevented by marrow therapy.

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[1]Mammalian Recovery Group.

[2]Chemical Protection and Enzyme Catalysis Group.

[3]D. G. Doherty and Francis Hacker, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 169–70.

[4]C. C Congdon, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 170–71.

13. Cell Physiology

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Subcellular Physiology (13.1, 13.3–13.6)

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E. C. Horn[b]
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[d]Visiting investigator from NATO.
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Cell Biophysics (13.8–13.11)

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S. P. Leibo[e]
Janice J. Schmidt

Molecular Physiology (13.2, 13.7, 13.12, 13.13)

W. D. Fisher
Tom Grizzard
G. B. Cline[a]

Cooperative AEC-NIH Development of an Ultracentrifuge (13.1–13.7, 13.13)

13.1 STUDIES ON SYNCHRONIZED *ASTASIA LONGA*[1]

G. M. Padilla P. A. Van Dreal

Introduction. – The problems involved in maintaining synchronized populations of the flagellate *Astasia longa* have largely been solved. As described in the previous reports,[2,3] techniques for continuous culture, constant monitoring of the changes in population density, and refeeding of mass cultures of cells have been developed. Synchronized populations are now being used in a series of studies concerning the role of physical factors on cell division.

Role of Carbon Dioxide Levels on Synchronized Cell Division. – As discussed in Chap. 13.3, one of the problems involved in the constant monitoring of the gas phase of cell cultures is the frequent loss of sterility with the use of electrodes. A possible alternative to the insertion of electrodes into the culture flask is the use of compressed gas mixtures bubbling through the growth medium and CO_2 and O_2 measurement in the exhaust gas stream. It was noted that in contrast to the normal situation, where a culture is not being gassed, the constant passing of air through the medium of synchronously dividing *Astasia* induced a delay of approximately 1.5 hr in the onset of the burst of division (see Fig. 13.1.1). When the air stream was supplemented with carbon dioxide to a concentration of 1.9%, the pattern of division was restored to the normal condition. A mixture of 5% CO_2 in air, on the other hand, reduced the time between the onset of the warm period and the beginning of the burst of division by approximately 0.5 hr. If air is bubbled throughout the cold period and a 5% CO_2–air mixture is introduced 15 min before the onset of the warm period, the burst of division is the same as if 5% CO_2 in air had been used during the entire cycle. Conversely, a similar result is obtained if 5% CO_2 in air is used throughout the cold period,

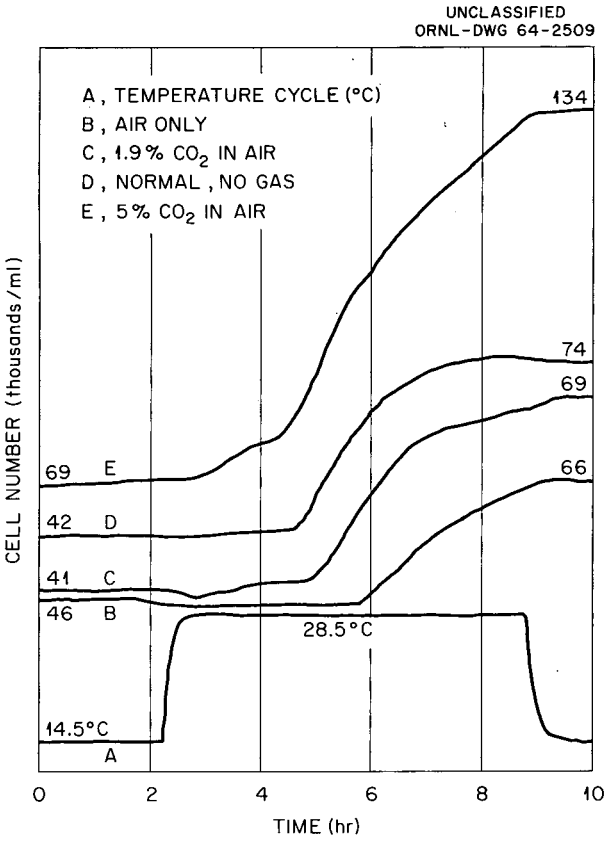

Fig. 13.1.1. Cell Burst Changes in Temperature-Synchronized *Astasia longa* Due to Various Gases Bubbled Through the Media.

45 min into the warm period, and then air alone is substituted. Changes in pH were also followed, and the largest deflection seen in the pH from the usual 6.9 value was of 0.1 unit. Such a small pH change has no effect on the growth of *Astasia*. These results suggest that CO_2 is required by some process taking place early in the warm period and that the effect is the reduction of the time required for cytokinesis. This phenomenon will be further investigated using isotopically labeled HCO_3^- or CO_2.

Effect of Low-Level X Radiation. – Following the demonstration that exponentially dividing *A. longa* undergo a division delay of 0.5 to 1.5 hr when exposed to x rays at dosages in the range of 100 to 400 r,[3] a series of determinations on the sensitivity of synchronously dividing cells to x rays were performed. The results indicate that as the cold period progresses, there is a gradual increase in the division delay from 0.02 to 0.1 min/r. Toward the end of the cold period and through the first $1\frac{1}{2}$ hr of the warm period the sensitivity increases, reaching a value of 0.4 min/r, at a time corresponding to the onset of mitosis. As the population passes through mitosis, the sensitivity to irradiation drops to 0.2 min/r. There is a second increase in sensitivity toward the end of the warm period. This probably corresponds in time to the resumption of nucleic acid synthesis of the cells. As the cold period begins, the sensitivity drops to near zero, as pointed out above. These results suggest the existence of alternating periods of radiosensitivity that are correlated with the cell cycle.

[1]AEC-NIH Development of an Ultracentrifuge.

[2]T. W. James, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp. 104–5.

[3]G. M. Padilla, T. W. James, and P. A. Van Dreal, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 142–45.

13.2 POLYRIBOSOMES FROM *TETRAHYMENA* – THEIR ROLE IN PROTEIN SYNTHESIS AND THEIR ALTERATION UNDER DIFFERENT ENVIRONMENTAL GROWTH CONDITIONS[1]

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G. B. Cline P. A. Van Dreal
G. M. Padilla

Introduction. – Pyrimidine starvation brings the ciliate *Tetrahymena pyriformis* to a nongrowing state. This state is characterized by decreased protein synthesis, a drop in the content of RNA and protein, and the cessation of DNA synthesis.[4,5] Upon replacement of the required pyrimidines there is a rapid increase in RNA synthesis rate that occurs prior to an increase in protein synthesis rate. This increase in protein synthesis precedes the initiation of macronuclear DNA synthesis, and the cell eventually divides. Based on cytochemical evidence, it appears that all the cells are synchronized in their biochemical response to this "shift up" in the growth conditions. The work reported here is part of a program to study the role of polyribosomes in protein synthesis and in growth control in this system.

Methods. – *T. pyriformis* (strain HSM) were grown on either a defined complete medium or a medium lacking the required pyrimidines. Cells brought to a nongrowing state by a 72-hr pyrimidine starvation were then exposed to the required pyrimidines for 40 min. Cultures of log phase, starved, and refed cells were lysed by exposure to a saturated indole solution in 5 m*M* Tris buffer, pH 7.5, with 0.5 m*M* $MgCl_2$.[6] All the cells ruptured, but the macronuclei were preserved unbroken. Prior to lysing by indole, the cells were pulsed with P^{32} orthophosphate and C^{14}-leucine. The suspension of broken cells was separated in the B-IV rotor in the zonal ultracentrifuge on a 10–30% sucrose gradient made up in the Tris-magnesium buffer. This gradient was supported on the rotor wall with a "cushion" of 55% sucrose.

Results. – Figure 13.2.1, top, shows the profile of 260-mμ ultraviolet absorbance from the suspension of logarithmically growing cells. The initial soluble peak at the starting boundary is followed by smaller peaks representing computed particles of 110, 160, 210, 250, and 280 S units, followed by an isopycnic "membrane fraction." The phase contrast microscope showed that the macronuclei and other large particulate material had passed through the 55% sucrose "cushion" and was adhered to the rotor wall. Electron micrographs show the 110S peak to constitute a rather homogeneous suspension of single particles, the 160S peak double particles, the 210S peak triple particles, etc. The peaks of 3, 4, and 5 particles occur predominantly as clusters and not in linear arrays.

The 72-hr pyrimidine-starved cells show a paucity of ultraviolet-absorbing peaks, especially the "membrane fraction" (Fig. 13.2.1, middle). In pyrimidine-starved cells that were given back their required pyrimidines (Fig. 13.2.1, bottom), one sees the reappearance of ultraviolet-absorbing material especially in the "membrane fraction" and along the trailing edge of the membranes.

Phosphorus-32 counts on the logarithmically growing cells show activity in the soluble and membrane fraction but no activity associated with the ribosome or polyribosome peaks. On the other hand C^{14}-leucine shows increased activity in each of the polyribosome peaks. The amount of radioactivity increases as an increasing function of the number of clustered ribosome particles.

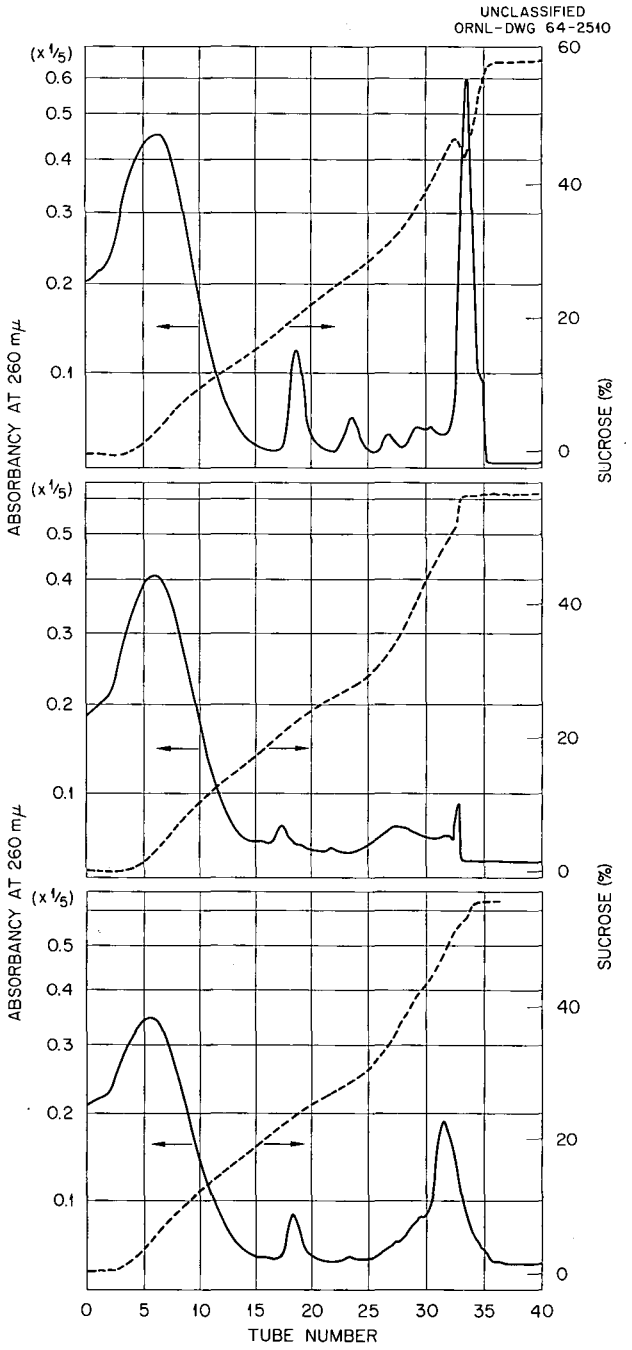

Fig. 13.2.1. Separation of the Particles from a Lysate of *Tetrahymena pyriformis* in the B-IV Zonal Ultracentrifuge Rotor. The top graph was obtained from logarithmically growing cells; the middle graph from 72-hr pyrimidine-starved cells; and the bottom graph from pyrimidine-starved cells that were refed their required pyrimidines for 40 min.

Discussion. – Apparently we are dealing exclusively with cytoplasmic polyribosomes, since the macronuclei have not ruptured in our preparations and do not contaminate the centrifugal separations. Further analysis of the isolated macronuclei should prove of interest in determining the origin of ribosomes. The electron microscope gives visual reassurance that the ultraviolet-absorbing peaks are polyribosomal in nature.

In *Tetrahymena*, 12 min elapse between introduction of labeled RNA precursors (H^3-uridine, H^3-cytidine) and their appearance in the cytoplasm.[7] Thus a 20-min pulse of P^{32} (used as an indicator of RNA synthesis) could not be expected to label much of the structural RNA of the ribosomes. In turn, C^{14}-leucine (used as a precursor and indicator of protein synthesis) would not be expected to contribute any noticeable label to the structural protein of ribosomes in this system. Therefore, we may assume that the cytoplasmic label, found after a 10-min pulse label with H^3-leucine, is nascent protein which is still attached to the polyribosome. These findings may be taken as an indication of the in vivo role of polyribosomes in protein synthesis and that the polyribosomes are the active sites of protein synthesis.

In the pyrimidine replacement experiment, the reappearance of ultraviolet-absorbing material in the 110S peak and along the trailing edge of the "membrane fraction" (Fig. 13.2.1, bottom) indicates that polyribosome material is being resynthesized 40 min after the "shift up" in growth conditions and is therefore partially reestablished prior to the increase in the rate of protein synthesis which occurs between 60 and 75 min.[4,5]

[1]AEC-NIH Development of an Ultracentrifuge.

[2]Cytochemistry and Cell Reproduction Group.

[3]Cell Growth and Differentiation Group.

[4]I. L. Cameron, *J. Cell Biol.* **19**, 12A (1963).

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

[6]J. W. Lyttleton, *Exptl. Cell Res.* **31**, 385 (1963).

[7]D. M. Prescott, *J. Histochem. Cytochem.* **10**, 145 (1962).

13.3 CONTINUOUS MONITORING OF CO_2 AND O_2 IN STERILE CULTURES USING NONSTERILE ELECTRODES[1]

P. A. Van Dreal

Previous work[2] in this laboratory reported the use of a sterile polarographic electrode to measure O_2 in temperature-synchronized cultures of the flagellate *Astasia longa*. These results showed that O_2 is utilized slowly during the cold period and at a much greater rate during the warm period. This suggested that monitoring CO_2 continuously over the cycle would also be of interest in examining the energetics of the cell burst. This was done using the Severinghous electrode to measure CO_2 and a polarographic electrode for O_2. One of the problems in using electrodes immersed in the culture media is the difficulty of maintaining sterility while changing electrode membranes and recalibrating. This problem has been avoided by bubbling 5% CO_2 in air through the media at slow rates in a closed culture vessel. The excess gas is led from the vessel in a gum rubber tube and allowed to equilibrate with a second vessel as shown in Fig. 13.3.1. This second vessel contains the O_2 and CO_2 electrodes immersed in triple-distilled water which was rapidly stirred on a magnetic stirrer.

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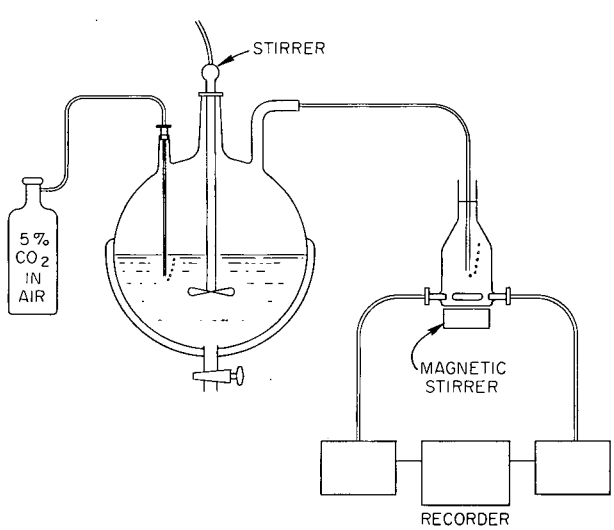

Fig. 13.3.1. Cell Culture System for Monitoring O_2 and CO_2 Changes with Nonsterile Electrodes.

If the gas flow rate is held at 120 cc/min, changes in O_2 consumption and CO_2 production can be measured against the background concentration of the compressed gas in the tank. An example of the results is seen in Fig. 13.3.2. Note that during the early part of the warm period, O_2 utilization is decreased while the CO_2 production is increased. The dotted lines indicate the change in level occurring in the same vessel and media without cells. These results suggest the possibility of a metabolic pathway change in the early part of the warm period. This method of monitoring gas opens up the possibility of controlling cultures on a demand basis according to their need for respiratory gases and for the elimination of gaseous waste products.

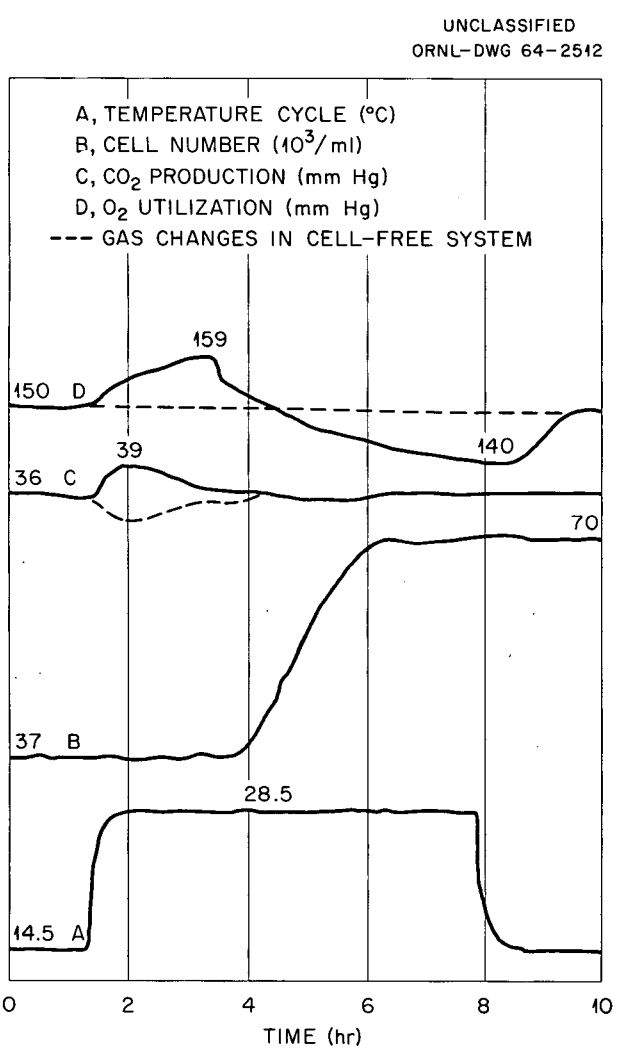

Fig. 13.3.2. Oxygen Utilization and CO_2 Production During the Synchronized Division Burst of *Astasia*.

[1]AEC-NIH Development of an Ultracentrifuge.

[2]T. W. James, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp. 104–5.

13.4 SYNCHRONIZATION OF CELL DIVISION IN *TETRAHYMENA PYRIFORMIS* WITH A REPETITIVE TEMPERATURE CYCLE[1]

G. M. Padilla I. L. Cameron[2]

Introduction. – Induction of cell division synchrony with a repetitive temperature cycle has been previously achieved with the protozoan flagellate *Astasia longa*. This flagellate, being a true autotroph, stands apart from most metazoan cells in its nutritional requirements. There arises, therefore, the implication that the technique of induction of cell division synchrony may be limited to cells of this type. If this is true, its applicability to other cells would be restricted and its usefulness as a research tool severely impaired.

In answer to this question, the synchronization procedure previously described[3] has been extended to the ciliate protozoan *Tetrahymena pyriformis* (strain HSM). This cell has certain advantages that make it a suitable organism for these studies: (1) its nutrition has been extensively studied and is now well defined, (2) its growth characteristics as a function of temperature are known, and (3) its cell cycle is currently being examined in several laboratories where single-cell techniques are being developed. Thus, *Tetrahymena* constitutes not only the organism of choice with which to extend the synchronization technique, but also in the assessment of the synchronization technique itself.

Results and Discussion. – Cultures of *T. pyriformis* growing on a 1% proteose peptone medium supplemented with 0.05% liver extract were placed on a variety of temperature cycles. The program which gave the most repetitive type of synchrony consisted of a 12-hr cycle with a 2.5-hr period at 27°C and 9.5 hr at 12°C. Figure 13.4.1 shows the type of synchrony achieved.

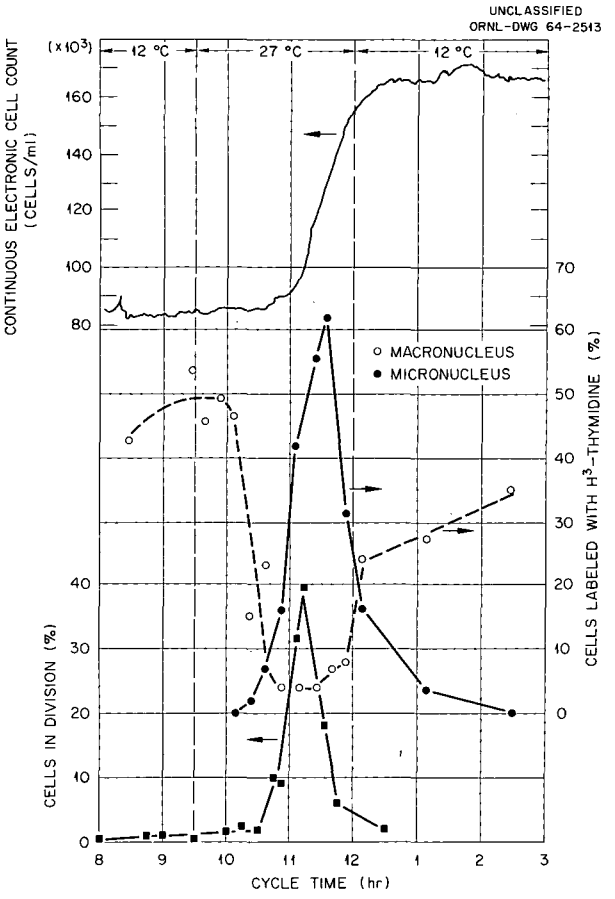

Fig. 13.4.1. Synchronization of Cell Division in the Ciliate *Tetrahymena pyriformis* (HSM). The abscissa shows the cycle time of the 12-hr cycle (2.5 hr at 27°C and 9.5 hr at 12°C), only portions of which are shown.

The upper tracing in the figure shows that the cells do not divide during the cold period (only portions of which are shown) but only in the last hour of the warm period. The burst of division, in which over 95% of the cells divide, continues slightly into the cold period. Compared with the generation time of 3 hr (at 27°C), these results indicate that there is a threefold acceleration in the doubling time of the population. To further assess the degree of synchrony in terms other than cytokinesis, the cells were given short time pulses with H^3-thymidine and radioautographed. The number of cells that incorporated the label in the macronucleus and micronucleus were scored. The results are also shown in Fig. 13.4.1. It can be seen that as the cells enter into the warm period, at least 50% of them show radioactivity in the macronucleus. Within 1 hr the number of cells with a labeled macronucleus drops to a few percent. Coincident with this decrease, the micronucleus becomes labeled, reaching a peak value of 61.7% in the middle of the burst of division. The macronucleus remains inactive during cytokinesis but resumes uptake of the label toward the end of the warm period, when the micronucleus becomes inactive. These results suggest that synchrony-inducing temperature shifts operate by alternately phasing macronuclear and micronuclear nucleic acid synthesis. Such interplays in the major synthetic patterns of subcellular structures point to the value of this system in studying the controlling mechanisms of cell division.

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[1]AEC-NIH Development of an Ultracentrifuge.

[2]Cytochemistry and Cell Reproduction Group.

[3]G. M. Padilla and T. W. James, *Exptl. Cell Res.* **20**, 401 (1960).

13.5 CARBOHYDRATE ANALYZER: IMPROVED SYSTEM FOR COLOR DEVELOPMENT[1]

J. G. Green N. G. Anderson

Introduction. – The general design and subsequent improvement of an automated carbohydrate analyzer have been reported previously.[2–5] The most unsatisfactory element of the system has been the color-development device which employed the segmenting of the mixed reagent stream with air bubbles, the layering of the aqueous sample on the dense reagent segment, and the development of color in an air-cooled heat exchanger. A different system which obviates the inherent disadvantages of this system is described here.

Materials and Design. – The boundary-layer diffuser is diagrammed in Fig. 13.5.1. Reagent and column eluant streams enter the diffuser and mix and interact during passage through the column composed of 3-mm-diam glass beads. The mixed stream is pooled in a short length of 6-mm tubing fixed to the base of the bead column. To prevent air locking at the pool and to minimize liquid bridging in the bead column, the pooling

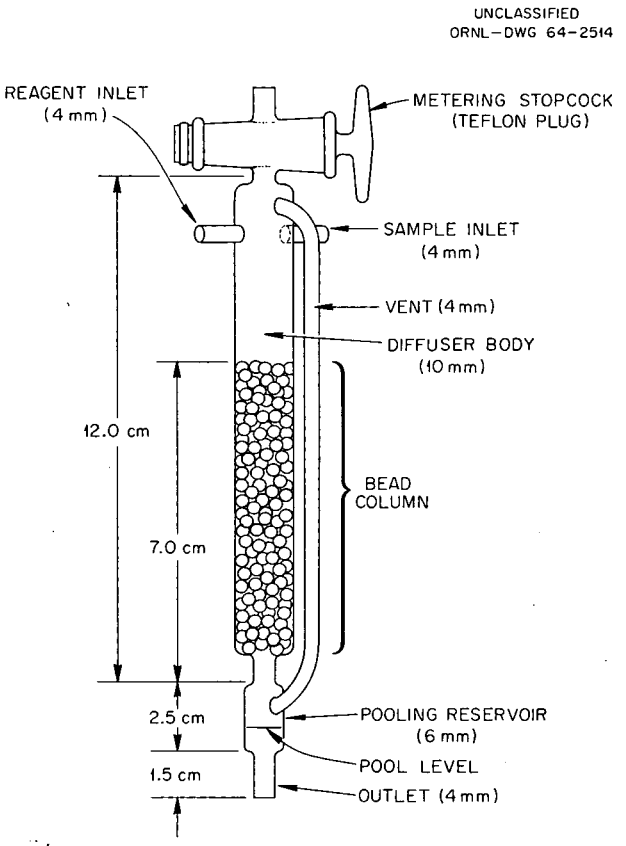

Fig. 13.5.1. Boundary-Layer Diffuser. The apparatus is fabricated from borosilicate glass tubing of the diameters indicated. Glass beads are 3 mm in diameter.

reservoir is vented to the top of the bead column. Air dissolved in the entering streams and, perhaps, other gases liberated during the process are vented to the atmosphere through a metering stopcock fitted with a Teflon plug. The mixed stream is conducted from the diffuser to the flow cell of the colorimeter or, more satisfactorily, is routed through a coil of Teflon tubing ($\frac{1}{8}$ in. OD × 0.030 in. wall) which is immersed in an improvised boiling water bath to prolong incubation and enhance color intensity.

Flow rates in the new system are 0.6, 3.0, and 1.1 ml/min for the phenol, acid, and column streams. The dispensing and column systems remain unchanged and retain the advantages of adjustable, precise flow control and air purging.

Results and Discussion. – Laminar flow and layering of the reagent and sample streams, which differ substantially in density, are mitigated by boundary-layer turbulence induced by the surfaces of the system. Adhesion of the fluids to solid surfaces coupled with intermolecular cohesion causes localized turbulence in the streams as they flow around the glass beads, and the mixing is further enhanced by convection currents established during the exothermic mixing of the streams. Diffusion during passage of the mixed stream further reduces any irregular proportioning which might occur. A bead column of given volume has minimal void space per unit of surface area; therefore, the effect of mixing and diffusion on the resolution of the system is negligible, as demonstrated in Fig. 13.5.2, where a comparison of elution curves from the two systems is made.

In the bubble segmented system, the aqueous sample layer was floated in the denser reagent stream; however, at acid concentrations above 88%, boiling occurred at the interface of the liquids. Varying back pressure from the intermittant boiling caused irregular flow from the phenol source which disrupted the base line in the manner indicated by the arrow in Fig. 13.5.2*a*, and the use of diluted acid caused reduced glucose sensitivity in the colorimetric system. The characteristic irregular flow pattern is not reflected in the trace in Fig. 13.5.2*b*, and the use of concentrated acid yields maximal glucose sensitivity in the new system with a reduced reagent consumption. Furthermore, the system is adaptable to incubation of the mixed stream in a constant-temperature bath, and preliminary investigation indicates that the required length of Teflon tubing in use may be only 20 ft.

The bleed rate of the diffuser gases is controlled manually at the present time; however, the system may be regulated automatically by a level-sensing device coupled to a metered solenoid valve.

Further study to define the optimum parameters of the boundary-layer diffusion system and to derive a refined system are in progress. In addition, experimentation to increase the applicability of the automated system continues.

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[1]AEC-NIH Development of an Ultracentrifuge.

[2]J. G. Green and N. G. Anderson, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1962*, ORNL-3352, p. 128.

[3]J. G. Green and N. G. Anderson, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 118.

[4]J. G. Green and N. G. Anderson, *The Joint NIH-AEC Zonal Centrifuge Development Program Semiann. Rept. June 30, 1963*, ORNL-3502, p. 75.

[5]J. G. Green and N. G. Anderson, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 148.

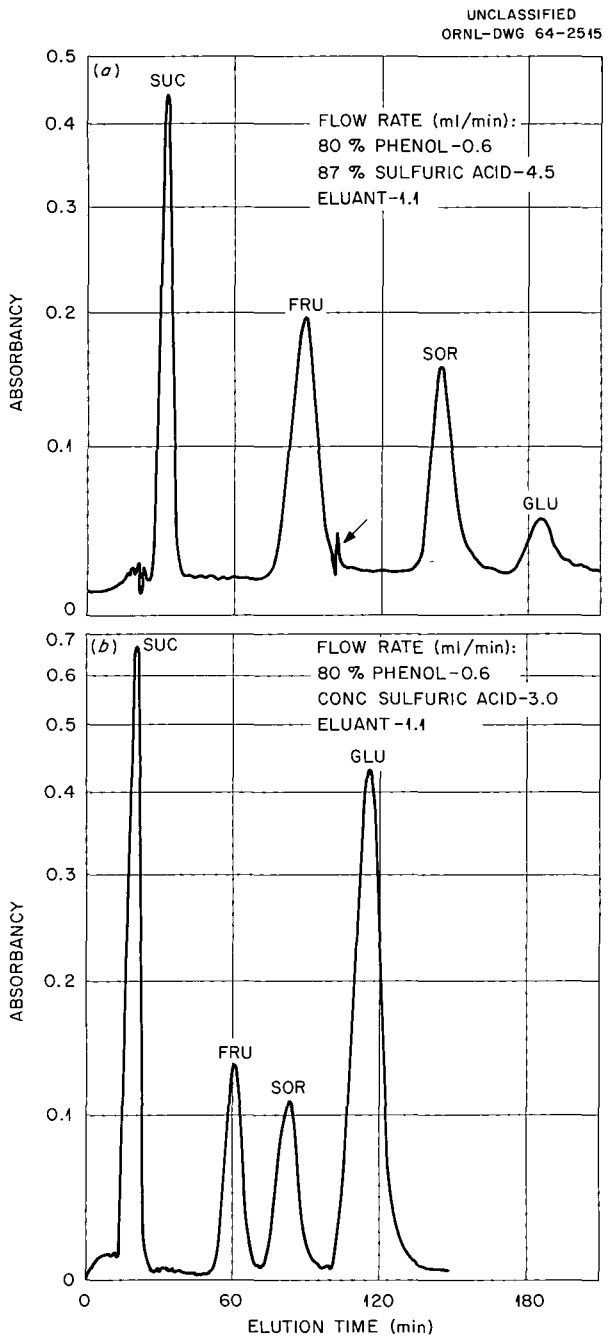

Fig. 13.5.2. Comparison of Elution Times for Two Systems. (*a*) Separation of 2.5 micromoles each of sucrose, fructose, and sorbose and 7.5 micromoles of glucose with initial color-developing system. Arrow denotes effect of interfacial boiling phenomenon. (*b*) Separation of 2 micromoles each of sucrose, fructose, and sorbose and 6 micromoles of glucose using boundary-layer diffuser color-developing system. Note superior sucrose and glucose sensitivity, reduced reagent consumption, and absence of interfacial boiling effect.

13.6 ISOLATION OF VIRUS PARTICLES FROM TISSUES[1]

N. G. Anderson C. L. Burger
W. W. Harris[2]

Introduction. – The mass of an average mammalian cell is 10^6 to 10^8 times the mass of a single virus particle. The isolation of trace amounts of virus, where the lower concentration limit is one particle per cell, would therefore represent from 1 to 100 million-fold purification. It is not feasible to consider such a difficult separation problem, unless it can be shown that there exist unique general properties of viruses which are distinct from those of the particulate components of cells. A review of the density and sedimentation properties of viruses and cell components has therefore been undertaken. The results of this work are shown in Fig. 13.6.1. The sedimentation and density data are compiled from literature sources and from data obtained in this laboratory. These results suggest that there exists a virus "window" in the sedimentation coefficient vs density in CsCl plot, which includes most viruses. Whether previously unisolated normal cell constituents will fall in this window remains to be seen. The problem therefore is to perfect means for the high-resolution separation of the constituents of cells (including viruses) on the basis of sedimentation rate and density. The specific experimental problem which we wish to approach is the isolation of virus particles from animal, and possibly human, tumors. The question of the relation of viruses to human tumors is an acute one. The definitive experiments which have established the field of animal viral oncology cannot be repeated in man. Oncogenic viruses are often species and strain specific, of low infectivity, and have long incubation periods. Negative biological experiments with extracts of human tumors are therefore inconclusive. The direct isolation of viruses from human tumors is a possible experimental route out of this dilemma.

Experimental. – The details of the development of sedimentation rate–density (S-ρ) techniques will not be presented here. The B-IV contained ultracentrifuge system has been developed to make the rate separation, while the density separations have been performed in angle head tubes

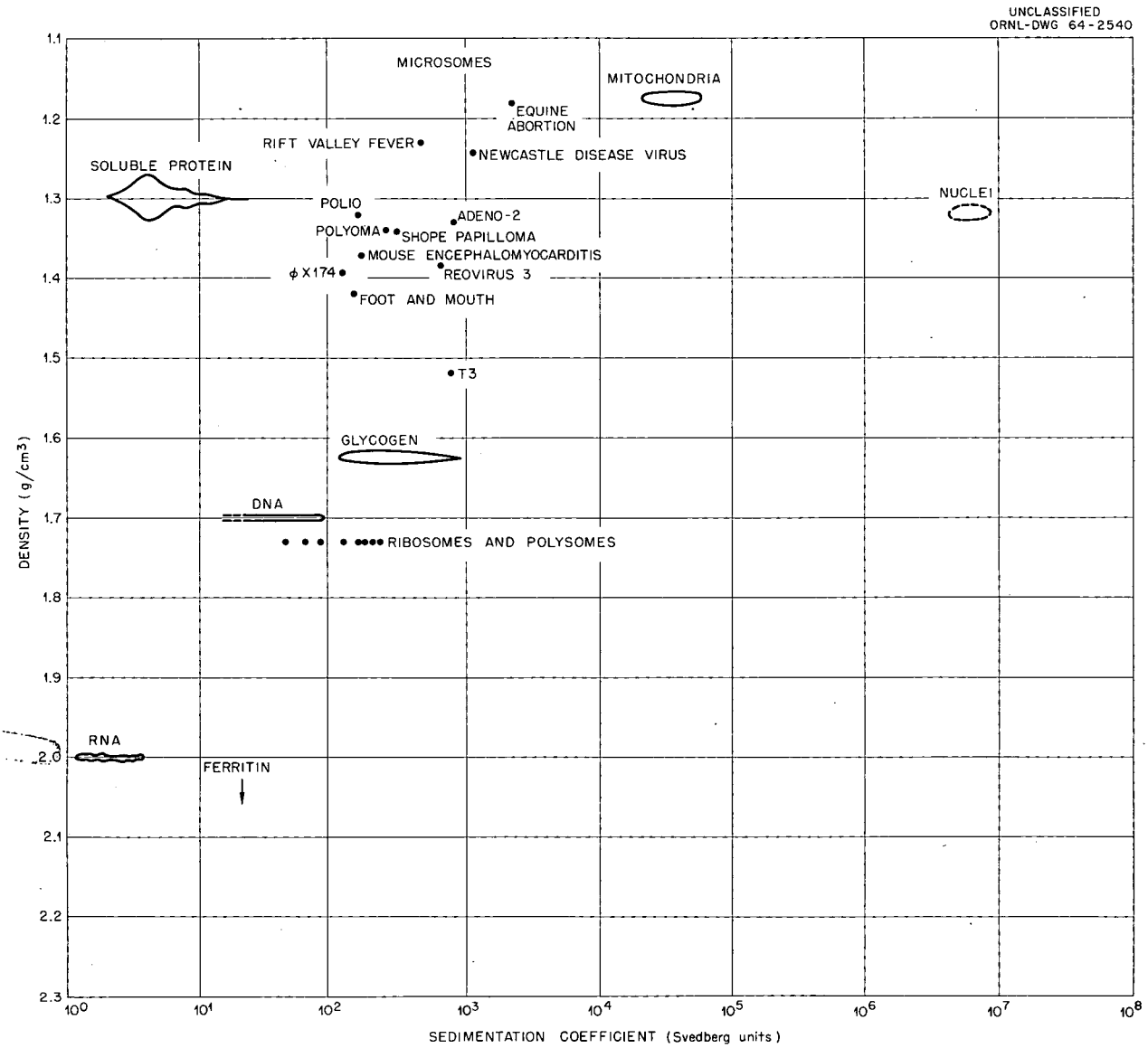

Fig. 13.6.1. Plot of Sedimentation Coefficients and Densities of Subcellular Particles and Viruses. Data collected from studies reported in the literature.

at the outset. Light scattering, ultraviolet absorption methods, and automatic recording of refractive index have been used to monitor the CsCl gradients.

Using T2 and T3 phage, added to rat liver homogenates, and Adenovirus 2 infected KB cells, it has been found that the S-ρ system is capable of isolating these viruses in a high state of purity. Determination of the lowest level at which particles may be detected is now in progress. Normal rat and mouse livers and spleens show only traces of material in the virus "window" area. As noted elsewhere, a few virus-like particles have been seen in normal mouse spleens in RF mice. In orienting studies on human spleen tissue from patients expiring with Hodgkin's disease and leukemia, many tailed and round myxovirus-like particles have been seen. The evaluation of these results must await extended studies on normal human control material.

Discussion. – The centrifugal methods for isolating particles in the size and density range of normal viruses from tissue homogenates has now been carried to the point where the feasibility of the underlying theory has been demonstrated. Definitive studies on virus-like particles in human tumors are now possible.

[1]AEC-NIH Development of an Ultracentrifuge.
[2]ORGDP.

13.7 THE B-IV ZONAL ULTRACENTRIFUGE[1]

N. G. Anderson E. F. Babelay[2]
H. P. Barringer[2] W. D. Fisher

Zonal centrifuges,[3–6] which are the preparative counterparts of the analytical ultracentrifuge, are being developed to cover a range of particle sizes which extends from whole cells to protein molecules. Of these, the series A rotors separate particles in the range of sizes visible in the light microscope, the series B rotors fractionate approximately the range of particles visible in sections in the electron microscope, while the high-speed C series rotors are designed for molecular separations. In practice, the particle-size ranges separable with these systems overlap. Twenty-four rotors have been designed and tested in the course of the present studies.

The basic problem has been to overcome the capacity and speed limitations inherent in swinging-bucket rotors, to accelerate large-volume gradients to the desired rotational speed without mixing, and to recover the gradient undisturbed at the end of this centrifugation. One solution to this problem has been to introduce the gradient and sample layer into the rotor during rotation, using centrifugal force to stabilize the gradient in sector-shaped compartments in the rotor. After the separations are completed, the gradients are recovered without bringing the rotor to rest.

The chief interest in the B series rotors has been in the large-scale isolation and purification of viruses for vaccines[6] and as a tool for isolating subcellular particles and for searching tumors for virus particles. For some of this work, especially the isolation of ribosome subunits, speeds up to 40,000 rpm are desirable. The B-II rotor previously described[5] proved to be destructively unstable in the region of 31,000 rpm. To reach higher speeds, a thorough study of the problem of rotor stability and a redesign of the core, upper bearing, and seal was indicated. This work has resulted in the development of the B-IV zonal centrifuge described here.

The pertinent physical constants of components of the drive system, rotor, upper bearing, and supporting frames were examined for possible sources of excitations which could cause the destructive high-amplitude vibrations. Careful measurements, theoretical studies, and detailed experimental investigations have led to a system which operates in a satisfactory manner at 40,000 rpm. Proximity detectors were installed inside the centrifuge vacuum chamber to study movement of the rotor during rotation. Studies at speeds near 31,000 rpm showed that a high-amplitude forward precessional mode of vibration was superimposed on the normal low-amplitude vibrations due to small rotor imbalances. The upper bearing was not sufficiently damped to absorb these vibrations.

A variety of upper bearings were tested in the development of an enclosed combination journal bearing–seal holder which provides the proper damping constant by shearing viscous oil between parallel plates. The bearing is self-aligning and is water cooled. The rotating seal which allows fluid to be pumped into and out of the rotor during rotation is completely contained to allow infectious materials to be handled without forming aerosols. It is of a unique design with three dams for retaining fluids while providing the proper preload on the seal faces without the application of external forces. Two dams on the upper surface of the seal slider prevent loss of fluids moving into and out of the rotors, while the fluid dam on the lower edge of the slider retains a circulating coolant which may also contain a disinfectant. Minimal cross leakage at speeds up to 40,000 rpm has been observed.

The cylindrical rotor contains a core which serves to prevent swirling during acceleration and deceleration and flow produced by Coriolis forces during loading and unloading.

The B-II core was designed with 2 constrictions to channel the gradient into the exit lines, and 36 septa to divide the rotor into as many compartments.[5] The latter design was made on the

mistaken assumption that movement of the fluid mass in the rotor contributed to rotor instability. The B-IV core has a simple four-vaned configuration and is constructed in one piece. The surfaces are positioned to minimize mixing due to laminar flow and still yield a maximum volume of fluid in the rotor. In addition, the core is self-supporting and does not load the rotor wall.

The rotor is driven by a modified Spinco model L ultracentrifuge with extended armor to accommodate the long B-IV rotor. For work with infectious agents or virus-like particles[7] which may be oncogenic, a Freon-tight enclosure is provided (Fig. 13.7.1). All controls, a new electronic system for continuous digital presentation of the integral of $\omega^2 dt$, and the electronic components of the spectrophotometer for recording the absorbance of the effluent stream are housed in cabinets outside the centrifuge.

The B-IV system has been used to isolate 100-mg quantities of ribosomes and ribosome subunits and lesser amounts of polysomes. Pulse-labeled cells rapidly broken in a French press have been run immediately into the rotor and the ribosomes and polysomes separated in 2 hr at 40,000 rpm.

As an example of the resolution which may be obtained, 70S ribosomes isloated from *E. coli* have been disassociated into 30 and 50S subunits and separated as shown in Fig. 13.7.2. The 50S peak in this experiment contained approximately 240 absorbance units of material [~45 mg (wet weight) of ribosomes]. It is evident that resolution of particles somewhat smaller than 30S is feasible in the B-IV system.

The B-IV system for making rate-zonal separations followed by isopycnic banding is being

Fig. 13.7.1. Complete Contained B-IV Zonal Centrifuge for Large-Scale Virus-Isolation Studies.

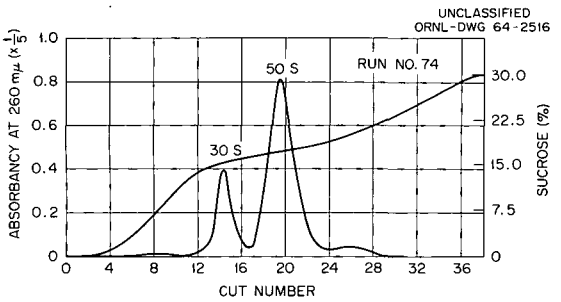

Fig. 13.7.2. Separation of 30S and 50S *E. coli* Ribosomes Prepared by Dissociation of 70S Ribosomes Previously Isolated in the Same Rotor. The gradient volume was 1000 ml, extending from 12 to 20% sucrose containing 0.01 *M* phosphate buffer, pH 7.5 and 0.01 *M* Mg^{2+}. A 30% sucrose solution was used as a cushion with 220 ml of buffer inboard of the sample layer. The separation was effected in $2\frac{1}{2}$ hr at 40,000 rpm (15×10^{10} $\omega^2 t$ for the entire run, including acceleration and deceleration). The tracing was obtained by displacing the rotor contents with 55% sucrose through a Beckman DB spectrophotometer containing a 1-mm flow cell and through a Waters model 35H refractometer.

used to attack the very difficult problem of isolating trace amounts of virus and virus-like particles from human tumors. The size and morphology of virus-like particles isolated from human Hodgkin's and leukemic spleen will be described in a subsequent report.

[1]AEC-NIH Development of an Ultracentrifuge.

[2]ORGDP.

[3]N. G. Anderson, *Bull. Am. Phys. Soc.* **1**, 267 (1956).

[4]N. G. Anderson and C. L. Burger, *Science* **136**, 646 (1962).

[5]N. G. Anderson, *J. Phys. Chem.* **66**, 1984 (1962).

[6]N. G. Anderson, *Nature* **199**, 1166 (1963).

[7]C. L. Burger *et al.*, *Proc. Soc. Exptl. Biol. Med.* **115**, 151 (1964).

13.8 FLOW OF WATER IN MULTICOMPARTMENTED CELLS AND TISSUES DURING FREEZING

Peter Mazur M. T. Harkrider[1]

Introduction. – As pointed out in the previous semiannual report,[2] a significant question has arisen regarding freezing injury in multicellular tissues: Is the formation of large ice crystals within individual cells of tissues incompatible with survival, as seems clearly to be the case with most single cells? The question has arisen because of observations by Sherman[3] and others that even when most or perhaps all cells of a tissue exhibit intracellular ice crystal artifacts, the tissue may still be capable of yielding viable transplants.

The previous report[2] outlined one approach to the question; that is to calculate the water contents of cells in multicellular model systems at subzero temperatures to determine the extent to which the water would be supercooled and, therefore, susceptible to freezing. General differential equations were derived for a three-layer system, and a preliminary numerical solution was presented. Solutions have now been obtained for conditions believed representative for vertebrate tissues.

Parameters. – The critical biological parameters affecting the numerical solutions are (1) the geometrical arrangement of the cells and the location of external ice, (2) the size of individual cells, (3) the permeability of the cells to water, (4) the temperature coefficient of the permeability constant, and (5) the molarity of the protoplasm.

A sampling from a histology text showed that the least dimension of mammalian cells ranged from 5 to 41 μ and the largest dimension from 6 to 65 μ. It was decided to consider cell layers 6 and 12 μ thick. The initial numerical solutions were applied to cells arranged in a three-layer sphere surrounded by ice; hence, fixing the layer thickness also fixed the volume of each layer and the area of the membrane surface between the layers. Values for the permeability of vertebrate cells to water (k_g) at room temperature (T_g) range from 0.21 $\mu^3/(\mu^2$ min atm) for the toad bladder[4] to 3.72 for intestinal mucosal cells of the rat.[5] The values selected for the numerical solutions were 0.3 and 3. Two published values[6,7] for the activation energies of water permeability in vertebrate systems are 9600 and 9800 cal/mole, equivalent to a temperature coefficient (b) of 0.065 $\deg^{-1}$, where b is defined by the equation

$$k = k_g e^{b(T-T_g)} .$$

The intracellular osmolarity in humans and other mammals is close to 0.3.

Results. – Figure 13.8.1 shows the percentage of water remaining in three concentric 12-μ-thick layers as a function of temperature when the cooling rate is (*a*) 1, (*b*) 10, and (*c*) 100°C/min and the permeability constant is 3. Or it represents the contents when the cooling rate is (*a*) 0.1, (*b*) 1, and (*c*) 10°C/min and the permeability constant is 0.3. The equilibrium curve (Eq.) represents the water contents of cells cooled infinitely slowly. The horizontal distance between a point on that curve and curves α, β, and γ (which refer to the outer, middle, and center layers respectively) indicates the extent to which the water in the layer is supercooled. In (*a*) the water contents of all three layers cooled at 0.1 or 1°C/min remain close to equilibrium and reach equilibrium at −3°C. Since the water in the layers is at no time supercooled more than 0.8°, it would not be expected to freeze. In contrast, if the multicellular system is cooled more rapidly at 10 or 100°C/min (*c*), the water becomes extensively supercooled. When, for example, the water contents in the three layers have been reduced to 60% of their initial values, the water would be supercooled 8, 21, and 30° respectively. But supercooling of 20° or more is extremely rare in biological systems, and so one would expect the water in the middle and central layers, β and γ, to freeze before their water contents had been reduced to 60%. In many cells, in fact, freezing occurs when the protoplasm becomes supercooled by some 5 to 15°.[8] Thus, one would predict a range of possibilities for the outer layer, α. If it could tolerate supercooling of only 5°, the water in it would freeze before more than 12% had left the cells. But if the water could supercool 15°, it would never freeze within the layer; instead the cells in the layer would become dehydrated.

In the intermediate case of Fig. 13.8.1*b*, where the cooling velocities are 1 or 10°C/min, we would expect all three layers to dehydrate rather than freeze internally since the water in the layers is never supercooled by more than 5°. On the other hand, it is clear that shifting the curves

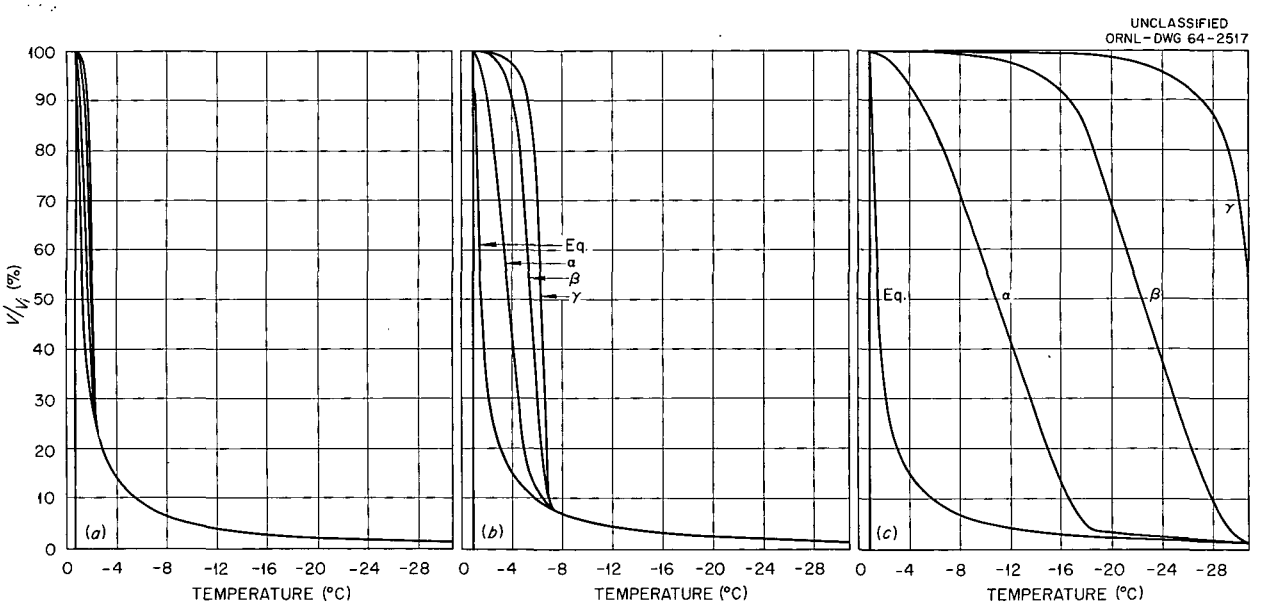

Fig. 13.8.1. Percentage of the Initial Water Content Remaining in the Three Layers of a Three-Layer Sphere Surrounded by Ice as a Function of Temperature. The curves labeled α, β, and γ refer to the outer, middle, and center layer, respectively, each layer being 12 μ thick. The curve labeled Eq. is the equilibrium curve (see text). (*a*) Cooling velocities = 0.1 and 1°C/min when k_g = 0.3 and 3 $\mu^3/(\mu^2$ min atm) respectively. (*b*) Cooling velocities = 1 and 10°C/min when k_g = 0.3 and 3 respectively. (*c*) Cooling velocities = 10 and 100°C/min when k_g = 0.3 and 3 respectively.

slightly to the right, as would occur with slightly more rapid cooling, slightly lower permeability to water, or a slightly reduced surface/volume ratio, would change the situation markedly. The outer layer would still dehydrate rather than freeze, but the inner layers would probably freeze before they fully dehydrated.

If the geometry and parameters chosen represent real tissues adequately, one would expect the following: At cooling velocities over 10°C/min most, and perhaps all, of the cells would contain ice; at cooling velocities of under 1°C/min few or none of the cells would be frozen; and at cooling velocities between 1 and 10°C/min, there would be a broad spectrum ranging from areas where no cells contained ice to others where all contained ice. If intracellular ice can be equated with death, the heterogeneity of response makes it clear that with cooling at 1 to 10°C/min, some cells could escape intracellular freezing and still be viable even when most of them contain ice and are therefore dead. If some cells are viable, the tissue itself may be viable, as judged by its ability to yield viable transplants. Higher cooling velocities would be expected to produce freezing in all cells and, hence, a nonviable tissue. Sherman found this to be true. Slower cooling, on the other hand, should produce fewer cells with visible internal ice and still a viable tissue. This point has not been investigated.

A more complete analysis of the situation in tissues will require numerical solutions for geometries other than spheres. Present plans are to obtain curves for cylinders and slabs.

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13.9 EFFECT OF THE WARMING RATE ON THE SURVIVAL OF YEAST CELLS FROZEN CENTRIFUGALLY

J. G. Green Peter Mazur

Introduction. – The survival of yeast cells frozen at cooling rates of 1°C/min or above appears to depend largely on whether or not ice forms within the cells. Low cooling rates (~1°C/min) favor high survivals because they cause the cells to dehydrate and as a consequence prevent intracellular crystallization. With higher cooling rates (>50°C/min), the cells do not have sufficient time to dehydrate and so they freeze internally.[1–3]

However, another phenomenon appears in rapidly cooled cells: their survival becomes dependent on the rate of warming and thawing, with rapid warming yielding the higher survivals.[1] Crystal size is known to decrease with increasing cooling velocities, and it has been hypothesized that (1) rapid cooling produces smaller crystals within yeast cells and (2) these small crystals, because of their higher surface free energy, grow to damaging size during slow warming, but not during rapid. The technique of centrifugal freeze-drying described previously[4,5] provided a means of testing this hypothesis since it permits reproducible extremely rapid freezing of cell suspensions. It therefore has been applied to determine the effects of varied warming rates on the subsequent survival of rapidly frozen unicellular yeast (*Saccharomyces cerevisiae*).

Experimental. – The yeast was cultured in liquid media,[1] isolated by centrifugation, washed three times with sterile distilled water, and resuspended in sterile water to a final concentration of about 2×10^8 cells/ml. In previous experimentation, the cell suspension was injected from a hypodermic needle into the spinning rotor containing liquid nitrogen; however, in the present study, 4 ml of the cell suspension was introduced into the rotor as an aerosol from a glass atomizer designed to give minimal evaporation of water from the cells during delivery and to produce small droplets. The freezing of the suspended cells was conducted in a rotor spinning at 3600 rpm in an International PR-2 centrifuge, since earlier unreported studies yielded best cell survival from this treatment. The frozen suspension, still in liquid nitrogen, was transferred to

an insulated cup to expedite transfer to the containers used in warming.

Two series of warming experiments were performed. In the first series, the frozen cells in a small amount of liquid nitrogen were poured into a beaker containing 1 liter of 0.08 *M* potassium phosphate plating diluent at temperatures of 5, 15, 25, 35, 45, and 55°C. To facilitate dispersion of the suspension during warming, the solution was stirred by a propeller agitator at a speed which caused sporadic vortex breakup.

Since the rate of warming of the cell suspensions could not be measured in these experiments, a second series of experiments was performed utilizing the following techniques.

1. The cell suspension was warmed to room temperature within the rotor.
2. The frozen suspension in liquid nitrogen was transferred to shell vials chilled to −196°C and warmed to room temperature in air.
3. The frozen suspension, free of liquid nitrogen, was ladled into shell vials chilled to −10°C. In one trial the cells were warmed to room temperature in air; but in subsequent trials, the vials were fixed to a copper block with silicone grease to increase the rate of warming.
4. The frozen suspension in liquid nitrogen was poured into plastic centrifuge tubes chilled to −196°C which were dipped into a 35°C water bath upon evaporation of the liquid nitrogen.
5. The frozen suspension in a small amount of liquid nitrogen was poured into a liter of plating diluent at 35°C.

The warming rates of the suspensions in trials 1 through 4 were monitored in duplicate by fine thermocouples placed in frozen suspension, and the thermocouple output was recorded continuously by a Leeds and Northrup Speedomax G recorder with dual chart speed attachment. The warming rate for trial 5 could not be measured directly, and estimated values are given.

Viability of the untreated control and treated suspensions was assessed by hemacytometer counting and suitable plating techniques.[1]

Results and Discussion. – Although droplet size of suspensions from a hypodermic needle decreases and becomes more uniform with increasing rotor speed, droplet size from aerosol delivery appears to be relatively independent of rotor speed and may be minimally affected by shearing effects at the liquid-nitrogen–air interface of the spinning rotor.

The results of the first series of experiments are shown in Fig. 13.9.1. The increase in survival with increasing bath temperature to 35°C can be attributed to the increased rate of warming, but the decrease in survival at higher temperatures may have been caused by damage to the cells resulting from exposure to the higher temperature and may be only indirectly or not at all related to the freezing treatment. The fact that no difference in survival appears in the 5 and 15°C treatments is probably related to the coalescence of the warming droplets into larger ice particles at the two temperatures.

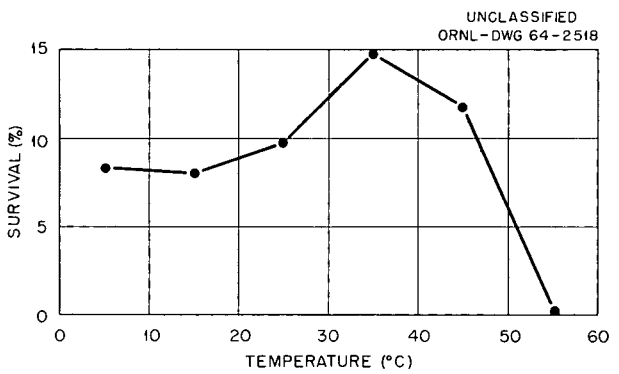

Fig. 13.9.1. Survival of Centrifugally Frozen Yeast Cells Warmed in 1-Liter Phosphate Diluent Baths at Different Temperatures.

The results of the second series of experiments, depicted in Fig. 13.9.2, confirm the fact that the survival of rapidly frozen cells is directly related to the warming rate. They strengthen the hypothesis that the survival of the cells is related to changes in crystal size and structure during the critical warming period. Since the exact freezing rate is not known, its effect cannot be independently assessed in this study.

Further study to measure the actual cooling rate of the suspension droplets and the warming

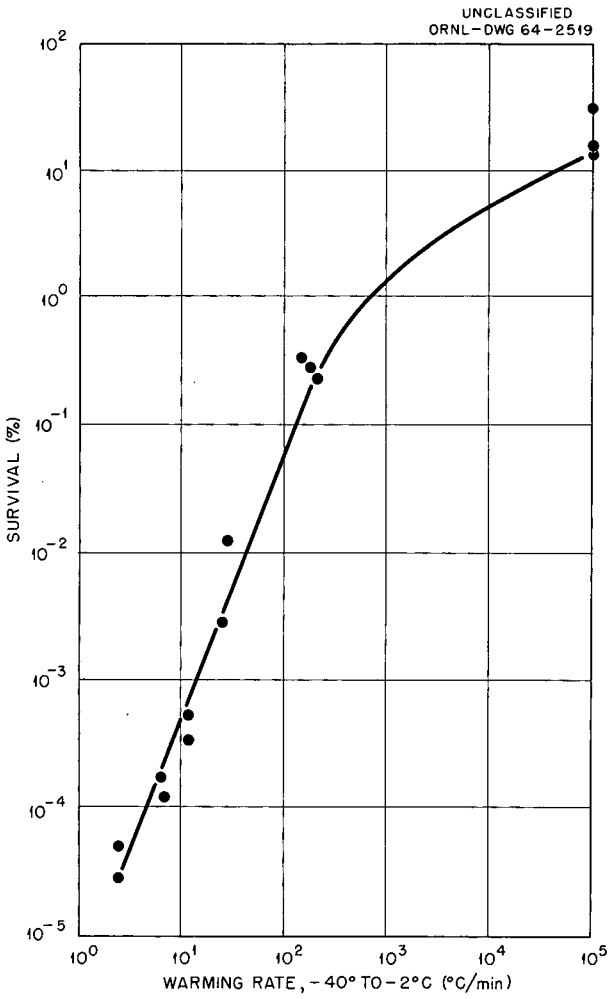

Fig. 13.9.2. Effect of a Measured Warming Rate on Centrifugally Frozen Cells.

rate in stirred solutions is in progress. In addition, an effort to increase the warming rate appears warranted.

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13.10 ROLE OF FREEZING IN THE FORMATION OF THYMINE DIMERS BY ULTRAVIOLET IRRADIATION

Walter Füchtbauer Peter Mazur

Introduction. – Dimerization of thymine upon ultraviolet irradiation at 2600 A occurs relatively easily in DNA and in di- and polynucleotides containing thymine, but thymine itself dimerizes in solution only when it is irradiated while frozen or dried. It has been suggested[1,2] that freezing acts by producing solid aggregates of thymine. But the question remains whether the dimerization results merely from the concentration of thymine or whether it involves more specific interactions between ice and thymine. The latter is suggested by the fact that the yield of dimers is much higher after irradiation of frozen solutions than of dry thymine.[3] To approach these questions, we are investigating the ultraviolet dimerization of thymine as a function of thymine concentration, state of water, and temperature. The first step was to determine the water-thymine phase diagram, consisting of the freezing-point curve and the solubility curve.

Experimental. – Solubilities (Fig. 13.10.1) were measured as follows:

1. Solutions were cooled to temperatures at which the thymine became saturated and solid thymine was present.
2. They were equilibrated, with stirring, in a constant-temperature water bath (±0.01°C) for at least 15 min, this time being sufficient to attain full crystallization.
3. They were then filtered at the same temperature through a 0.45-μ Millipore filter held in a Millipore high-pressure filter holder.
4. The concentration of thymine in the filtrate was determined spectrophotometrically.

Values obtained by approaching the desired temperature from below agreed well with those by approaching from above.

The freezing-point curve (Fig. 13.10.2) was obtained by cooling solutions with different concentrations of thymine and reading the temperature of the stirred solutions to ±0.001°C with a Beckmann thermometer during cooling. The temperature of the supercooled solution elevates as soon as freezing occurs and then remains constant for about 10 min; this plateau was taken to be the freezing-point temperature. It was noticed

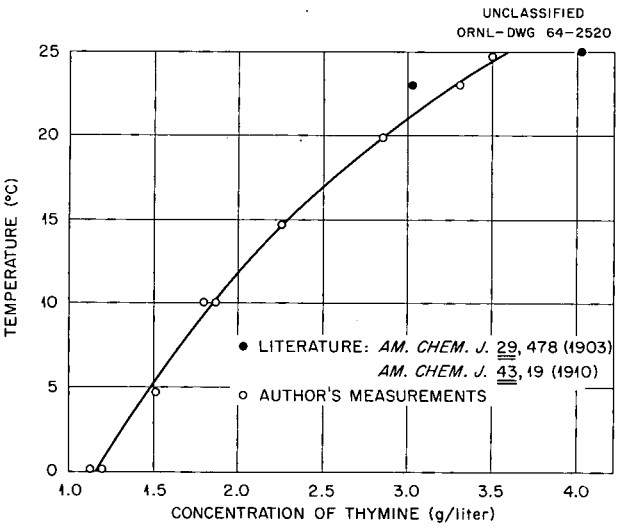

Fig. 13.10.1. Influence of Temperature on the Solubility of Aqueous Thymine Solutions.

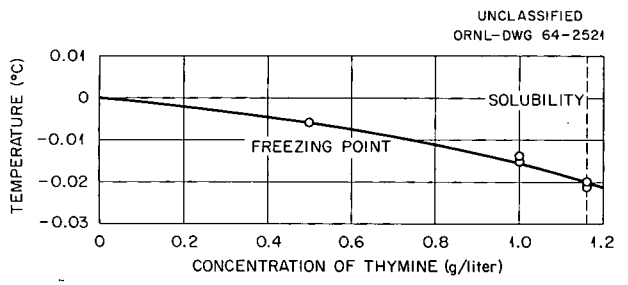

Fig. 13.10.2. Freezing Points of Aqueous Thymine Solutions.

that the more the solution supercooled, the higher and more reproducible was the observed freezing point; hence, the solutions were always supercooled by 2 to 3°C by slowly cooling them. The freezing points obtained this way were reproducible.

Discussion. – The only previously available data on the solubility of thymine[4,5] are indicated in Fig. 13.10.1. The observed freezing points coincide well with the values calculated using van't Hoff's formula, since the saturated thymine solutions are dilute enough to behave like ideal solutions. The intersection of the solubility and freezing-point curves is the eutectic point, which lies very near 0°C. Below the eutectic point the solution consists of ice and solid thymine only. Accordingly, temperatures below –0.02°C will not alter the concentration of thymine. It remains to be seen whether they alter the process of ultraviolet dimerization.

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13.11 WATER AND ICE RELATIONSHIPS IN BACTERIOPHAGE T4

S. P. Leibo Peter Mazur

In recent years the relationship between subzero temperatures and biological systems has received increased attention, but relatively few studies have been concerned with the resulting chemical and physical changes in cellular systems.[1] Fewer yet have investigated the alterations of isolated and intact macromolecular systems under these conditions, although there is evidence that such alterations do take place in proteins[2–5] and viruses.[6–8]

Experiments have been initiated to determine the effect of freezing and thawing on the infectivity, structure, and chemistry of bacteriophage T4. In this way it is hoped to provide a bridge between the freezing of isolated molecular systems and freezing of cells. As an adjunct, the water and osmotic relations of bacteriophage are also being investigated.

Methods and Results. – One hypothesis of freezing injury attributes the damage of cells to the concentration of salts which accompanies the freezing of dilute solutions.[9] In order to test this suggestion with respect to bacteriophage, phage T4 was suspended in solutions of various salts at their eutectic concentrations at 25°C for various times. The salts were selected to provide a wide range of eutectic points (–3 to –55°C) and pH (4.3 to 11.3). The infectivity of T4 was assayed on *Escherichia coli* B by the double-agar-layer technique and was compared to controls

held in nutrient broth. There was less than 20% loss of infectivity after exposure for 3 hr to H_2O, 1.2 *m* NH_4HCO_3, 4.6 *m* NH_4Cl, 1.4 *m* $MgSO_4$, 1.1 *m* KNO_3, and 5.2 *m* NaCl; but essentially 100% loss of infectivity followed exposure to 3.8 *m* $CaCl_2$, 2.9 *m* $MgCl_2$, and 0.5 *m* Na_2CO_3. After 30 hr at 25°C, infectivity decreased considerably in phage exposed to H_2O, NH_4Cl, and NaCl.

Since bacteriophage equilibrated in concentrated solutions are known to be inactivated following rapid dilution[10] (a situation comparable to that which would occur during the rapid thawing of phage frozen in a salt solution of concentration less than that of the eutectic), preliminary experiments have been performed to determine the "osmotic sensitivity" of phage T4. Samples of T4 were equilibrated for 20 min at 25°C in various concentrations of KNO_3 and in water. Aliquots of these suspensions were then diluted 1000-fold with water as rapidly as possible (of the order of 2 or 3 sec) and were assayed for infectivity. The results in Fig. 13.11.1 show a progressive decline in survival with increasing concentration of the equilibration medium. In contrast, control phage equilibrated in H_2O and rapidly diluted with H_2O gave about 90% survival of infectivity compared to phage equilibrated in nutrient broth and diluted with broth. Further, phage equilibrated in 3.0 *m* KNO_3 and rapidly diluted with 3.0 *m* KNO_3 yielded survivals of about 70% of phage T4 equilibrated in and diluted with H_2O. (Less than 0.05% of phage T4 equilibrated in 4.0 *m* NaCl survived a rapid dilution with H_2O.) The results with KNO_3 differ from those of Anderson *et al.*[10] They reported an abrupt drop in survival above a critical concentration, whereas we find that survival of T4 decreases continuously with increasing concentration of KNO_3 in the equilibration medium. Experiments to explore these discrepancies are being continued.

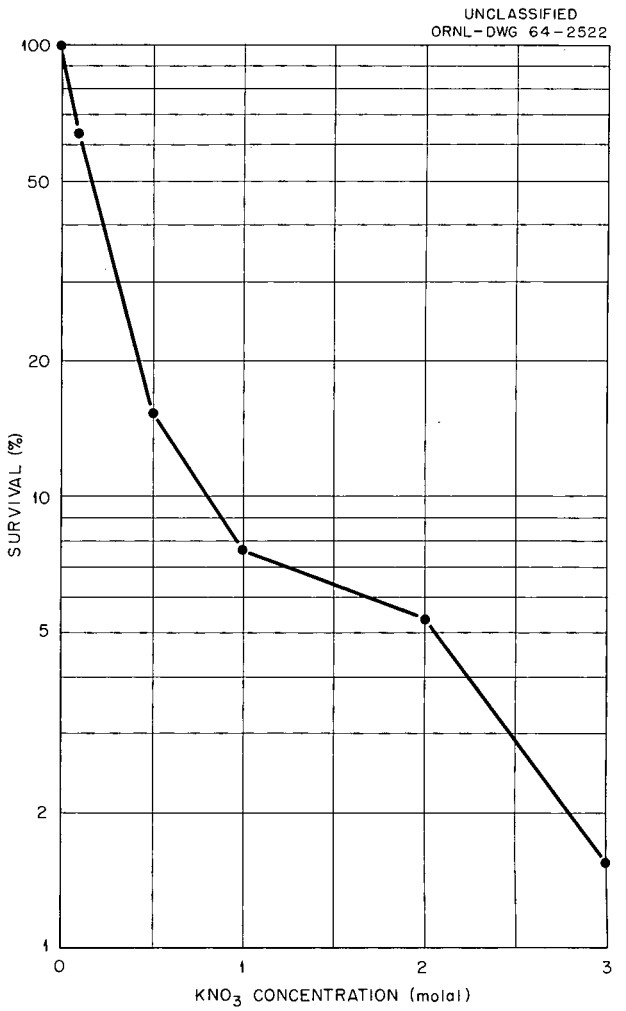

Fig. 13.11.1. Percent Survival of Bacteriophage T4 as a Function of the Molal Concentration of KNO_3 in Which the Phage Were Equilibrated for 20 min Before 1000-Fold Dilution With Water. Control suspensions were equilibrated in H_2O before dilution with water.

Preliminary experiments have been performed to determine the effect of exposure to various subzero temperatures on the infectivity of phage T4 suspended in 1.15 *m* KNO_3 (the eutectic concentration). By use of freezing procedures previously described,[11] it has been found that the sequence of rapid cooling–rapid warming to and from −20, −30, or −76°C is much less harmful to bacteriophage T4 than slow cooling–slow warming to and from these temperatures. For example, approximately 8% of T4 survive rapid cooling to −30°C and rapid warming, while only 0.8% survive slow cooling and slow warming.

The infectivity titers in these experiments, however, were somewhat variable, and there were suggestions that the value and variability of the titer may have depended on the dryness of the agar plates and other conditions involved in the assay. Therefore, the following experiment was carried out: Plastic culture dishes were filled with 30 ml of 1.5% nutrient agar and allowed to dry for various lengths of time in a single layer

at room temperature. Assays of infectivity of T4 suspended in nutrient broth or 1.15 *m* KNO_3 and held at room temperature and of T4 frozen and thawed from –76°C in 1.15 *m* KNO_3 were carried out on plates of varying dryness. Appropriate dilutions of T4 were pipetted either directly onto the surface of the bottom agar layer, over which was poured soft agar containing bacteria, or into tubes containing melted soft agar plus bacteria at 46°C, the entire mixture then being poured onto the bottom agar layer. The results in Fig. 13.11.2 show that when phage in either broth or nitrate was pipetted directly onto the bottom agar layer, the apparent number of infective phage per milliliter decreased with increasing dryness of the plates; but when phage in either broth or KNO_3 was mixed with bacteria in the liquid state, even at 46°C, there was no obvious decrease in infectivity with increasing plate dryness. A similar situation was also found for phage frozen

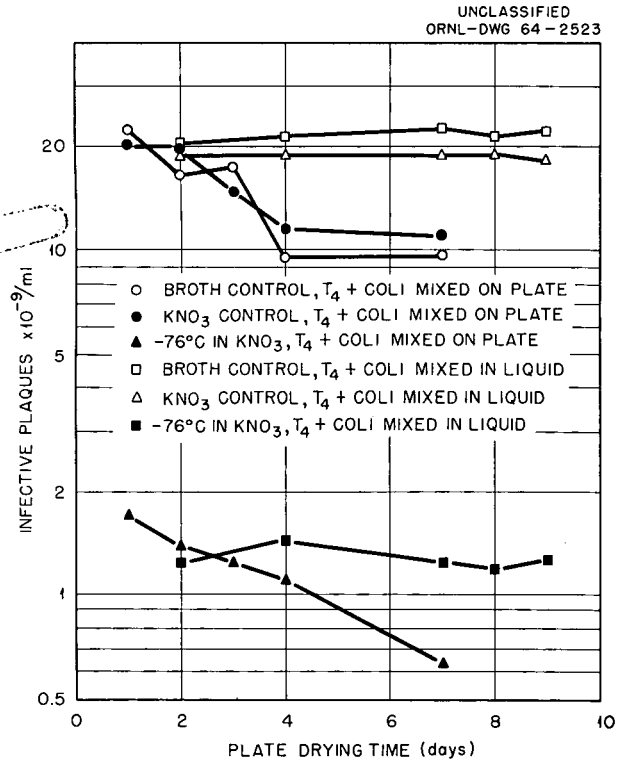

Fig. 13.11.2. Number of Infective Phage per Milliliter as a Function of the Number of Days That the Plates on Which the Assays Were Made Had Been Dried at 25°C. The various symbols indicate the treatments and the conditions of assay.

and thawed from –76°C. It should also be noted that when the phage were pipetted directly onto the bottom layer, the variability between replicates of the same sample and between replicates of the same treatment increased with increasing dryness of the plates used for the assay. In view of these last findings, the results of the preliminary experiments on the freezing and thawing of phage T4 in KNO_3 must now be reevaluated.

Discussion. – The ability of bacteriophage to survive a wide variety of salts and salt concentrations provides a useful tool in the study of water and ice relationships in phage. For example, it will be possible to subject phage to various subzero temperatures either in the frozen or nonfrozen states. With data from various sources,[12–15] the internal head volume of the T-even phages is calculated to be 2.48×10^8 A^3, and the volume of phage DNA is 2.14×10^8 A^3. These figures suggest that approximately 3×10^7 A^3 of the internal volume of phage is occupied by water. It is hoped that the combination of studies on "osmotic shock" and on freezing and thawing will aid in determining the nature and state of this water and what role it plays in the configuration of the DNA in intact phage.

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13.12 MESSENGER-RNA-CONTAINING FRACTION FROM *E. COLI* B

G. B. Cline W. D. Fisher
Elliot Volkin[1]

Introduction. – A new species of protein-synthesizing particle has been found in several biological systems. These particles, called polyribosomes, or polysomes, have been isolated from rabbit reticulocytes,[2,3] rat hepatic cells, HeLa cells, fertilized sea urchin eggs, a ciliate (*Tetrahymena*), three species of bacteria (*Bacillus megaterium, Salmonella typhimurium,* and *Escherichia coli*), and, finally, cellular slime molds. The *E. coli* system has not been characterized beyond the observation of labeled particulates in a range of sizes above 70S.

The involvement of the bacterial cell membrane in protein and RNA synthesis is well documented.[4,5] The membrane has been thought of as a structural base for the arrangement and/or containment of cellular enzymes, cytochrome, and protein synthesizing systems. Starting from the "membrane" fraction, it should be possible to isolate structural elements, such as membrane-bound components, if they are present and if adequate methods of isolation are employed.

By use of the system employed previously for the study of rapidly labeled messenger RNA in T2 infected *E. coli* B,[6] attempts were made to fractionate the heavy "membrane" fraction to liberate particles containing protein-synthesizing units directed by pulse-labeled T2 messenger RNA. This report discusses a partial purification of large subcellular units in the zonal ultracentrifuge to which P^{32}-labeled T2 mRNA remains attached in low-magnesium buffers.

Results and Discussion. – Pressure-disrupted *E. coli* previously infected with T2 and pulsed with P^{32}-orthophosphate were put on sucrose density gradients of liter volume in the zonal ultracentrifuge. Cells were disrupted in either phosphate-magnesium or Tris-magnesium buffers where the magnesium concentration was low enough to cause dissociation of all ribosomes into 30-S and 50-S subunits. A typical absorbancy profile is shown in Fig. 13.12.1. The fast-moving peak (*M*) at the right represents an isopycnic "membrane" fraction, while the small peak to the left (*SM*) represents a slightly less-dense isopycnic "submembrane" fraction. The specific activity of acid-precipitable T2 DNA-like RNA in the two "membrane" fractions is similar but is up to 50 times the specific activity of this same RNA on the 50-S subunits. This represents a five- to tenfold enrichment of this fraction over previous investigations.

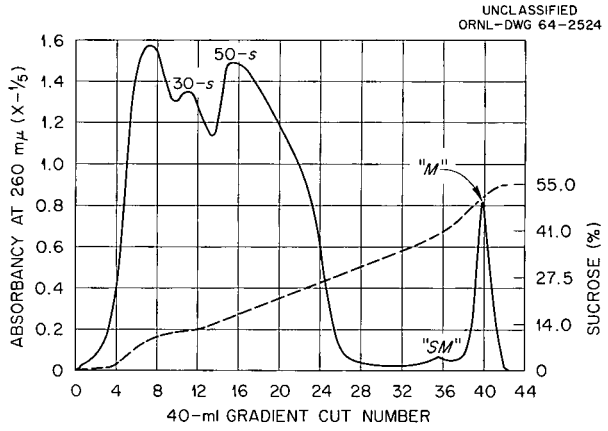

Fig. 13.12.1. Optical Density Profile of Zonally Separated Particulates from Pressure-Disrupted *E. coli* B. Cells were disrupted in 0.01 *M* phosphate–10^{-3} *M* $MgCl_2$ buffer and separated on a 10–30% sucrose gradient made up in phosphate-magnesium buffer. "*M*" is isopycnic membrane fraction, "*SM*" is isopycnic "submembrane" fraction, and 30-*S* and 50-*S* represent ribosome peaks. Wet weight of cells before disruption was 3 g.

Pelleting both "membrane" and "submembrane" fractions from the sucrose and allowing them to stand in the cold for 60 hr caused 70-S particles to appear. Treatment of these fractions, before dissociation, with ribonuclease did not cause visible peaks to appear as schlieren patterns. Desoxycholate (DOC) treatment of the 317-S fractions had little if any effect on reducing the size of the large component and producing a smaller peak.

The appearance of heavy particulates from *E. coli* disrupted in the presence of dilute magnesium and deoxyribonuclease indicates the presence of cell fractions intermediate between the whole cell and the smaller ribosomal subunits. The presence of pulse-labeled T2 DNA-like RNA in these large fractions, along with the appearance of 70-S breakdown units, strongly suggests the existence of functionally organized ribonucleoprotein particles on these large particulates.

Attempts are presently being made to determine, by means of electron microscopy and analysis in the analytical ultracentrifuge using ultraviolet optics, the effect of more-exhaustive ribonuclease treatment. The possible effects of trypsin, DOC, and other detergents will be studied in an attempt to characterize more fully and enrich the "submembrane" fraction from T2-infected cells.

[1]Nucleic Acid Chemistry Group.

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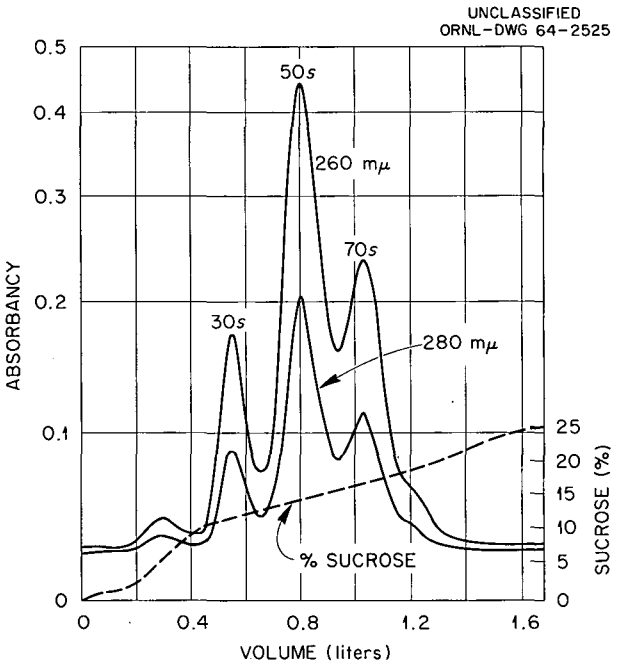

Fig. 13.13.1. Separation of *E. coli* Ribosomes After $2\frac{1}{2}$ hr at 40,000 rpm ($150{,}000 \times 10^6\ \omega^2 t$) in the B-IV Zonal Rotor in a 1-Liter Sucrose Gradient (13–20% with 0.01 *M* Tris pH 7.5 and 0.1 *M* Mg^{2+}). 220 ml of buffer was used as overlay and 500 ml of 25% sucrose as a cushion. The rotor contents were displaced through a 2-mm flow cell in a Beckman DB spectrophotometer and a Milan 34H recording refractometer and the outputs recorded on a Brown recorder.

13.13 ISOLATION OF RIBOSOMES IN THE B-IV ZONAL ULTRACENTRIFUGE[1]

W. D. Fisher G. B. Cline
Thomas Grizzard

Introduction. – The B-II zonal ultracentrifuge[2] has been modified to the B-IV system (see Chap. 13.7) to permit operation at 40,000 rpm. Studies have been made of the separation of *Escherichia coli* ribosomes to assess the usefulness of this rotor in separating biological particles in the 30 to 100s size class. Concurrently, analytical and biochemical work is also being done on the isolated fractions (see Chap. 15.2).

Results. – Figure 13.13.1 shows the separation in sucrose gradient of *E. coli* ribosomes from a ribosomal pellet prepared by differential centrifugation. The major peaks have nominal sedimentation coefficients of 30, 50, and 70s and contain 15, 60, and 30 mg (wet weight) of ribosomes respectively. Approximately two to three times this amount of material can be separated without loss of resolution. The fractions are homogeneous when rerun in the analytical ultracentrifuge.

Discussion. – The B-IV zonal rotor system provides a rapid method for obtaining milligram quantities of materials of ribosomal size by rate-zonal centrifugation.

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14. Nucleic Acid Chemistry

Nucleotide Chemistry		Molecular Biology	
W. E Cohn	A. Jeannine Bandy	F. J. Bollum	G. E. Houts
		B. K. Zimmerman	Marsha M. McClelland
		Masahiko Yoneda[a]	Patricia A. Williams

[a]Visiting investigator from abroad.

14.1 BIOSYNTHETIC POLYDEOXYNUCLEOTIDES

F. J. Bollum Patricia A. Williams
G. E. Houts Masahiko Yoneda

The *terminal*-deoxynucleotidyl transferase has been separated from the *replicative*-deoxynucleotidyl transferase of calf thymus gland.[1] We are using the terminal transferase to prepare a series of deoxypolynucleotides of completely known sequence, structure, and chain length. Sequence and structure are known by the mode of preparation:

$$\text{pXpYpZ} \ldots + n\ \text{DATP} \longrightarrow \text{pXpYpZpA}_n + n\ \text{PP}_i\ ,$$

where pXpYpZ is any known initiator sequence having the required 3′-OH group required for initiation.[2] Chain length is controlled by limiting n. The polymers are isolated in 100-mg quantities in 70–80% yield after a simple purification by alcohol precipitation and dialysis.

The two series (T_mA_n, with m = 3–9 and n = 100 or 300; 14 polymers) prepared and analyzed in detail so far are shown in Table 14.1.1.

Several additional polymers, not yet analyzed in detail, are also listed. These polymers, and others to be prepared, are to be used in studies on DNA replication, DNA transcription, and translation of the known polydeoxynucleotide sequences into polypeptide chains.

[1]Masahiko Yoneda, Patricia A. Williams, G. E. Houts, and F. J. Bollum, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 156.

[2]F. J. Bollum and G. E. Houts, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1960*, ORNL-2913, p. 108.

Table 14.1.1. Analysis of Polydeoxyadenylates[a]

Description	n = 100 (Theor.)[b]			n = 300 (Theor.)[b]		
	$E(P)$[c]	n (actual)[b]	$S_{20,w}$	$E(P)$[c]	n (actual)[b]	$S_{20,w}$
T_3A_n	9.27	180	3.70	8.01	283	4.69
T_4A_n	8.37	135	3.15	8.73	252	4.43
T_5A_n	8.98	102	2.31	8.04	258	4.08
T_6A_n	8.22	85	2.18	8.52	308	4.15
T_7A_n	8.61	109	2.28	7.98	376	4.12
T_8A_n	9.50	70	2.18	9.05	362	4.06
T_9A_n	9.00	99	2.37	9.63	296	3.89
Av	8.84	111	2.59	8.56	305	4.20

[a]Polymers $X_{3-8}A_{300}$, A_{300}, DNA·A_{300}, T_6A_{50}, T_6A_{100}, T_6A_{250}, T_6A_{600} have also been prepared but not yet analyzed in detail.

[b]n (theoretical) is estimated from polymerization recipe, $n = \frac{\text{micromoles monomer}}{\text{micromoles initiator}}$; n (actual) determined by base analysis.

[c]$E(P)$ is absorbancy at 260 mμ per micromole of phosphate.

15. Nucleic Acid Enzymology

Elliot Volkin	
Microbial Systems (15.1–15.4, 15.8, 15.9)	**Developmental Biochemistry (15.5–15.7)**
Elliot Volkin W. E. Barnett J. X. Khym Eric Schram[a] R. K. Fujimura C. G. Mead M. Helen Jones	F. J. Finamore N. G. Sansing[b] Rose P. Feldman A. H. Warner[b]
[a]Visiting investigator from abroad. [b]USPHS Fellow.	

15.1 BIOCHEMICAL TRANSFORMATIONS OF BACTERIOPHAGE T2 DNA

Elliot Volkin M. Helen Jones

Introduction. – In the previous report[1] it was shown that during the transfer of parental T2 DNA to progeny DNA, there was about a 20% reduction in the contribution of parental hydroxymethyldeoxycytidylic acid (HMC) relative to the contribution of the other three deoxynucleotides. This report describes experiments designed to test whether there is specificity involved in the elimination from transfer of certain HMC residues and whether the biochemical phenomenon is related to the extent of genetic recombination.

Results. – Parental T2, uniformly labeled with P^{32}, was used to infect *Escherichia coli* B in a phage to bacterium ratio of 4:1. After 60 min the infected cells were lysed with chloroform, and the progeny phage were isolated and purified. The progeny T2 DNA contained 20% less parental HMC relative to the other parental deoxynucleotides. These progeny phage were then used to infect *E. coli* B in a 4:1 ratio, and after 60 min their progeny T2 were collected and purified. It was observed that there was little, if any, further relative reduction in HMC transfer. These results indicate that some specific HMC residues are eliminated during the transfer of parent to progeny DNA. The data also point out that the relative contribution of parental HMC to progeny DNA is not merely due to the unequal reutilization of parental DNA nucleotide breakdown products.

In the last report[1] it was stated that when unlabeled T2 infect uniformly P^{32}-labeled host, the very early T2 progeny contain HMC with a markedly elevated P^{32} content relative to the other deoxynucleotides. When these progeny phage are used for infection, their progeny DNA contains a much greater reduction in parental HMC than the 20% loss previously observed. Thus it appears that many of the same HMC sites that were incorporated very early were preferentially excluded from transfer, rather than a random deletion of some HMC deoxynucleotides.

Mosig[2] demonstrated that the frequency of recombination between T4 mutants is much higher

in *E. coli* strain K(λ) than in strain B. When K(λ) is infected with uniformly P^{32}-labeled T2, the following results are obtained. A greater extent of breakdown of parental DNA to acid-soluble fragments is obtained – about 20% compared with 5 to 8% during *E. coli* B infection. A much greater reduction in parental HMC transfer to progeny HMC is observed – about a 35% loss compared to the transfer of deoxyadenylic (A) and deoxythymidylic (T) acids. It is of interest that there is less contribution of parental deoxyguanylic acid (G), relative to A and T, into progeny DNA when K(λ) is used as host.

Summary. – In the transfer of parental T2 DNA to progeny T2 DNA, a specificity in the loss of some HMC residues, relative to the other three deoxynucleotides, can be demonstrated. Biochemical transformations of parental T2 DNA are exaggerated in the host K(λ) compared with the host *E. coli* B. A correlation between this increase in the observed chemical alterations and the relative increase in genetic recombination of bacteriophage in the host K(λ) may exist.

[1]Elliot Volkin and M. Helen Jones, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963,* ORNL-3498, p. 158.

[2]G. Mosig, *Genetics* **48**, 1195 (1963).

15.2 ANALYTICAL STUDIES ON RIBOSOMES

Eric Schram

Introduction. – During the past six months our work has been concerned with the study of the structural proteins of *Escherichia coli* ribosomes. Although much information has recently been gained about the role of ribosomes in protein synthesis, far less is known about their actual mode of formation and especially about the synthesis of their structural proteins. Experiments were therefore performed with the ultimate goal of gaining some insight into the specificity and the mechanism of synthesis of these proteins.

Results. – *Separation of 30S and 50S Ribosomal Subunits by Zonal Ultracentrifugation.* – For our preparative separations, use was made of the B-IV zonal rotor developed by N. G. Anderson and his group (see Chap. 13.7). Coli were grown in the presence of S^{35}-sulfate so that the proteins could be selectively traced by means of their radioactivity. The purest ribosomal subunits were obtained by first isolating the 70S ribosomes in a 13 to 20% sucrose gradient and by rerunning this fraction in the same gradient after dissociation at low magnesium concentration (see Fig. 15.2.1).

Separation of Ribosomal Proteins by Disk Electrophoresis. – Polyacrylamide columns were introduced for the separation of ribosomal proteins, and satisfactory results were obtained when a buffer of rather low pH was used. The patterns obtained for the 30S and 50S subunits both show about a dozen peaks, which appear consistently in all our samples but which are quite different for both kinds of particles (see Figs. 15.2.2–15.2.4). Only very slight differences could be observed between the patterns for "native" and "derived" 50S particles. No differences were observed between the ribosomes from cultures at rest and those from exponentially growing cultures. The amino acid analysis of these several samples is now under way.

Radioactivity Determinations. – Although not apparent from the ultraviolet scanning of our preparative density-gradient centrifugations nor from the patterns obtained with the analytical ultracentrifuge, contamination of the 30S particles by the overlapping soluble protein peak could easily be inferred from the radioactivity curves. This contamination occurred only when the particles were not prepared from formerly isolated 70S particles and was encountered even when

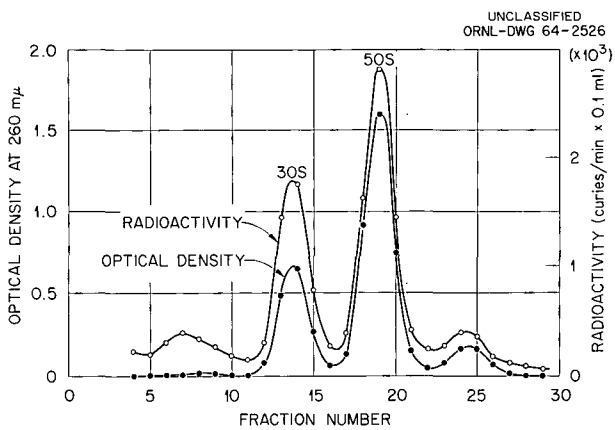

Fig. 15.2.1. Sucrose Gradient Separation of Ribosomes.

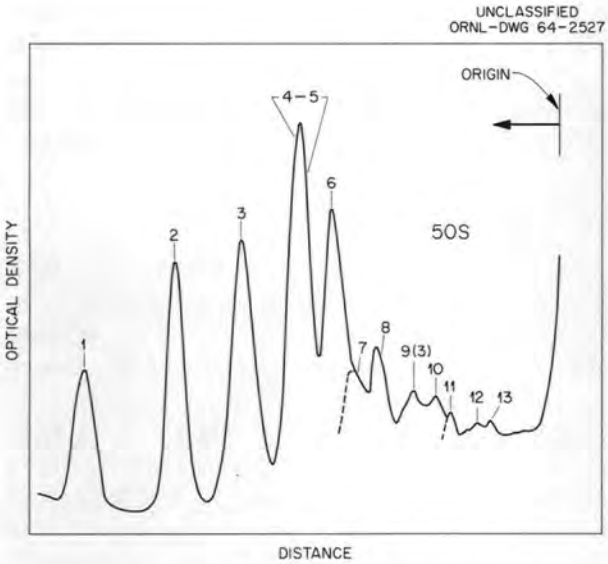

Fig. 15.2.2. Disk Electrophoresis of 50S Proteins.

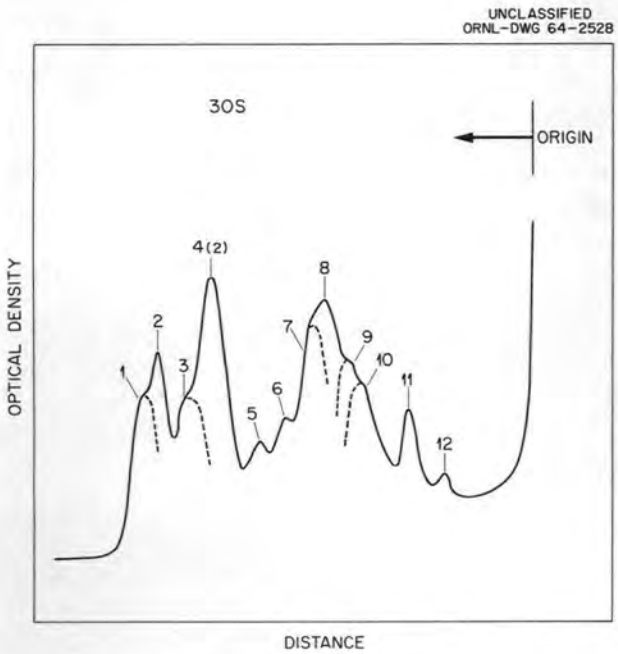

Fig. 15.2.3. Disk Electrophoresis of 30S Proteins.

starting from a partially purified ribosome pellet. The specific radioactivity (radioactivity vs RNA content) of the 30S subunits appears to be 50% higher than that of the 50S. After chasing, the same ratio is observed, but the specific radio-

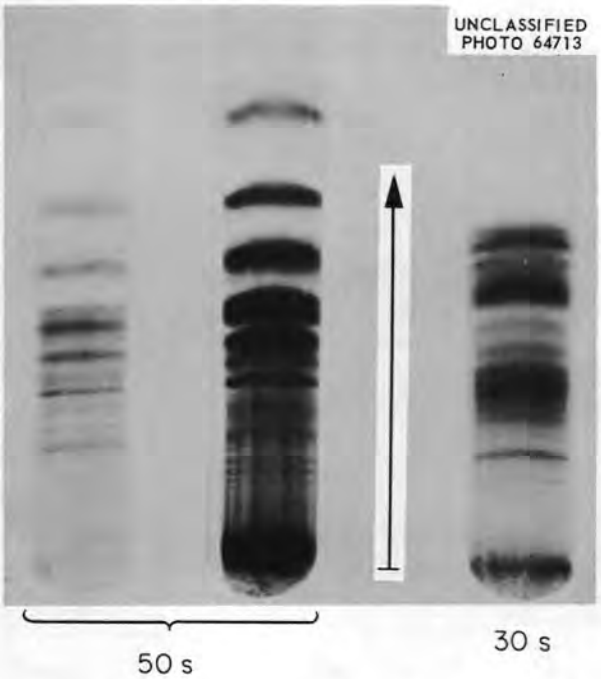

Fig. 15.2.4. Disk Electrophoresis of 50S and 30S Proteins.

activity of the native 50S particles is then found to be much higher than that of the derived ones. The radioactivity, before and after chasing, of the several proteins isolated by disk electrophoresis is now being studied.

Discussion. – Green and Hall[1] have observed differences in the physiological activity and RNA composition of native and derived ribosomal subunits. Such a difference does not appear clearly from our electrophoresis patterns, but our radioactivity data would at least imply the absence of an equilibrium between both types of particles. Moreover, the higher specific activities found in native 50S after chasing would mean that these particles are actually derived from the 70S, contrary to what has sometimes been anticipated. The higher specific radioactivities found in the 30S as compared to that of the 50S could be ascribed to a higher protein content if it can be confirmed that the proteins from both the 30S and 50S contain the same percentage of cystine and methionine.[2] This view is further supported by the fact that a lower 260/235 ratio is consistently found for the 30S.

We hope that the radioactivity measurements which are now under way on the proteins separated by disk electrophoresis will enable us to get a

deeper insight into the above facts. They will also show whether some of the proteins identified undergo a higher turnover than others.

[1]M. H. Green and B. D. Hall, *Biophys. J.* 1, 517 (1961).

[2]P. F. Spahr, *J. Mol. Biol.* 4, 395 (1962).

15.3 EVIDENCE FOR DEGENERACY AND AMBIGUITY IN INTERSPECIES AMINOACYL-sRNA FORMATION

W. E. Barnett K. B. Jacobson[1]

Introduction. – The role of transfer or sRNA in protein synthesis has been elucidated and amply reviewed. Distinct sRNA species for the different amino acids have been separated, and amino-acid-specific aminoacyl-sRNA synthetases have been partially purified. As adapter molecules the various sRNA's are direct intermediates involved in positioning amino acids in a correct linear sequence for polymerization into protein molecules. A prerequisite in assuring the correct positioning of an amino acid, therefore, is that the activated amino acid–synthetase complex recognize the correct sRNA molecule for aminoacyl-sRNA formation. Thus, each species of sRNA must have a site or recognition which distinguishes it from all other species of sRNA. In this report, recognition unit will refer to that series of nucleotides which is responsible for amino acid specificity, and references to coding will refer exclusively to these recognition units.

Data are presented in this report which indicate that the code used in aminoacyl-sRNA recognition units is not universal and which demonstrate interspecies degeneracy and ambiguity.

Results. – *Escherichia coli* sRNA has been charged with L-phenylalanine using synthetases from both *E. coli* and *Neurospora crassa*. Figure 15.3.1 shows that homologously charged *E. coli* sRNA chromatographs as a single peak on methylated albumin. Heterologously charged phenylalanyl-sRNA, however, consists of at least three distinct species. Following chromatography, the radioactive amino acid has been quantitatively re-isolated and identified as L-phenylalanine. Thus the observed chromatographic differences are not due to (1) selection by the heterologous enzyme for a contaminating radioactive amino acid or (2) conversion of phenylalanine to some other amino acid and subsequent aminoacyl-sRNA formation.

It was found that L-alanine competes with phenylalanine for peak II sRNA. Figure 15.3.2 shows that the presence of an excess of C^{12}-L-alanine during heterologous charging of *E. coli* sRNA with C^{14}-phenylalanine results in the loss of peak II radioactivity.

Discussion and Summary. – These observations support the notion that the coding used to maintain amino acid specificity in the synthetase-sRNA recognition unit system is not universal. The *Neurospora* phenylalanyl-sRNA synthetase selects

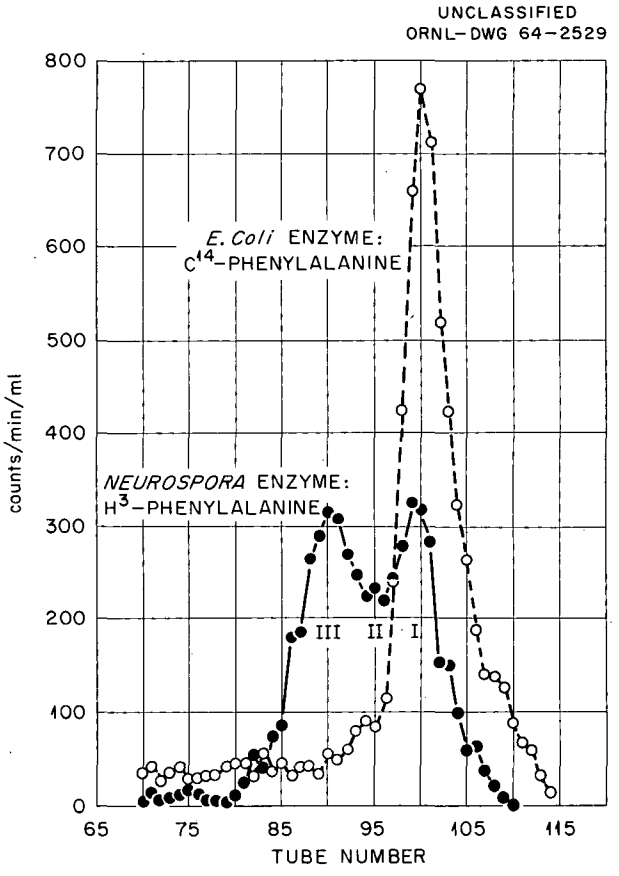

Fig. 15.3.1. Methylated Albumin Chromatography Elution Profiles of *E. coli* Phenylalanyl-sRNA Formed by Homologous and Heterologous Synthetases.

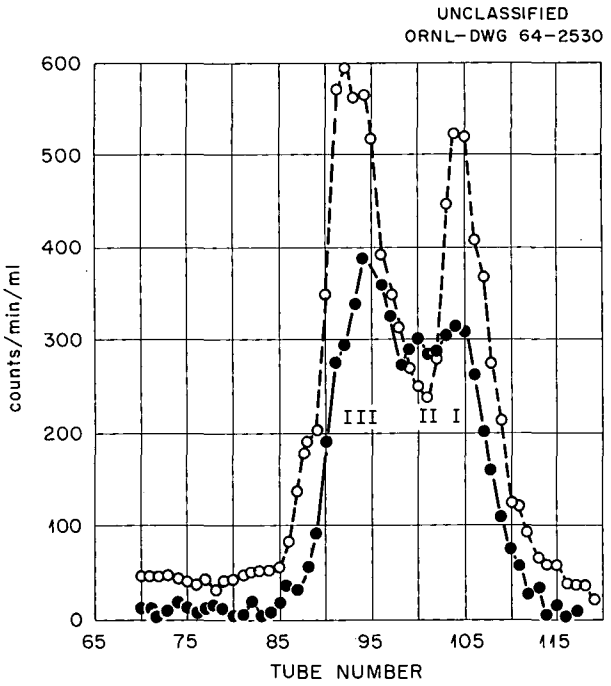

Fig. 15.3.2. Elution Profiles of *E. coli* Phenylalanyl-sRNA Charged by *Neurospora* Aminoacyl-sRNA Synthetases in the Absence and Presence of C^{12}-L-Alanine. H^3-Phenylalanyl-sRNA was formed in the absence of alanine (solid line), and C^{14}-phenylalanyl-sRNA was formed in the presence of 0.5 μM C^{12}-L-alanine per milliliter (dashed line). The radioactive aminoacyl-sRNA's were reisolated and cochromatographed on methylated albumin.

E. coli sRNA of peaks I, II, and III as acceptor molecules for phenylalanine. The *E. coli* enzyme, on the other hand, when confronted with the same selection of sRNA's, recognizes only one species (peak I) as phenylalanine sRNA. Such interspecies coding degeneracy would not be expected were coding identical in the two species. The apparent ability of peak II sRNA to accept either L-phenylalanine or L-alanine from the synthetases of *Neurospora*, but not from the homologous enzyme, indicates interspecies coding ambiguity and further supports lack of universality.

[1]Enzymology group.

15.4 ENZYMIC CONDENSATION OF OLIGODEOXYRIBONUCLEOTIDES

C. G. Mead

Extracts of *Escherichia coli* infected with T2 phage have been examined for an enzymic mechanism responsible for the fragmentation and recombination of T2 phage DNA during the vegetative phase after infection. The condensation of low-molecular-weight oligodeoxyribonucleotides with longer polydeoxyribonucleotides (sonicated DNA) was utilized as an assay system. The assay employs DEAE paper disks for detecting gross differences in chain length of polynucleotides. Classes of polynucleotides of different chain lengths can be selectively eluted from DEAE paper disks with different concentrations of NaCl in the presence of urea. The high-speed supernatant fraction of extracts of *E. coli* infected with T2 phage is capable of catalyzing the condensation of P^{32}-labeled oligodeoxyribonucleotides from T2 phage DNA with sonicated T2 DNA. After removal of endogenous nucleic acids by DEAE column chromatography, an absolute requirement for sonicated DNA, ATP, and Mg^{2+} is demonstrable. This enzymic activity is presently being purified of nucleases to enable the examination of the nature of the product.

15.5 OLIGONUCLEOTIDE NATURE OF ACID-SOLUBLE RNA IN AMPHIBIAN EGGS

F. J. Finamore Rose P. Feldman

Introduction. – Our recent discovery of acid-soluble RNA in ovarian eggs of *Rana catesbieana*[1] and our observation that marked changes occur in this fraction during embryonic development of *Rana pipiens*[2] prompted us to investigate further the chemical nature of this peculiar RNA fraction.

Methods. – The total RNA from ovarian eggs of *R. catesbieana* was prepared by a phenol procedure and purified as described previously.[1] The RNA was subjected to DEAE cellulose column chromatography using linear gradients of ammonium formate in the presence of urea.[3] The contents of the peak tubes were pooled, and to remove urea, each

fraction was diluted threefold and applied to small DEAE columns. After a thorough wash with water, the oligonucleotides were stripped from the columns with 1 *M* NH_4HCO_3. The NH_4HCO_3 was removed by treatment with Dowex 50-H^+, and after filtration, the samples were neutralized and concentrated under reduced pressure. Aliquots of each sample were used for analysis of total phosphorus, terminal phosphorus, acid-labile phosphorus, and nucleotide-nucleoside ratios.

Results. – Figure 15.5.1 shows a typical separation of total RNA in the presence of urea on DEAE columns. Six acid-soluble peaks are separated from each other and from the larger acid-insoluble RNA. The double peak obtained in the acid-insoluble region is characteristic of the ovarian-egg RNA preparations.

Table 15.5.1 shows a characterization of each peak by (1) ratio of terminal phosphorus to total phosphorus, (2) ratio of labile phosphorus to total phosphorus, (3) ratio of nucleoside from 5′-hydroxyl end or 3′-hydroxyl end to nucleotide residue, and (4) extent of hydrolysis and products obtained upon alkaline hydrolysis. The structures depicted for each peak are based on these values and are considered only as tentative.

One common feature of peaks 2 to 6 is that no nucleosides are liberated upon alkaline hydrolysis. This suggests that no free 3′-hydroxyl end groups are present in any of these oligonucleotides.

It is quite clear that peaks 1, 4, and 5 are mononucleotides, tetranucleotides, and pentanucleotides respectively. Peak 6 shows a nucleotide/nucleoside value indicative of pentanucleotides, but the terminal phosphorus/total phosphorus indicates extra terminal phosphorus. This is substantiated by analysis of alkaline hydrolysates of the peak, and since no labile phosphorus is present, the pentanucleotides of peak 6 contain most probably both 3′- and 5′-phosphoryl end groups.

Peaks 2 and 3, however, show a peculiarity that does not permit their ready identification: they each contain labile phosphorus. That these two peaks are not mixtures of nucleoside di- and triphosphates is shown by the fact that they are not converted to nucleosides on treatment with a bacterial alkaline phosphatase preparation known to contain not only phosphomonoesterase activity but di- and triphosphatase activity as well.

Upon alkaline hydrolysis, peaks 2 and 3 are not quantitatively converted to mononucleotides. Only about 60 and 20%, respectively, of the total optical density units (at 260 mμ) are recovered as mononucleotides from Dowex 1–chloride columns; the remaining optical density units of peak 2 can be recovered from the columns with 0.01 *N* HCl, but stronger HCl solutions are necessary for recovery of the remaining units of peak 3. These alkali-resistant fragments contain labile phosphorus,

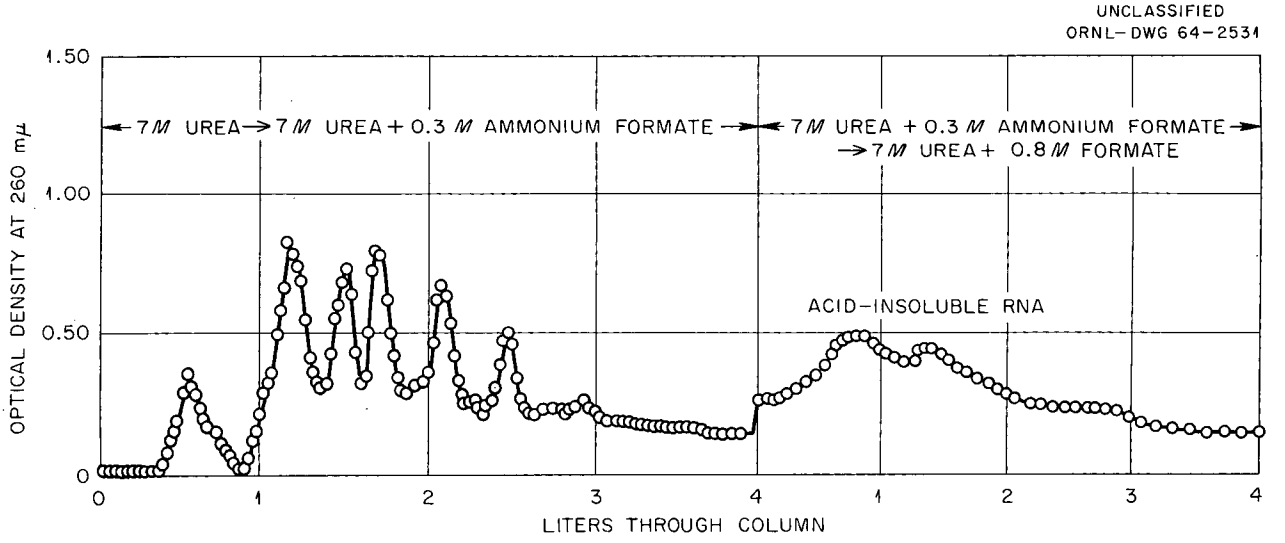

Fig. 15.5.1. Cellulose Column Chromatography of Ovarian Egg RNA.

Table 15.5.1. Analysis of Oligonucleotides Obtained from the Acid-Soluble RNA Fraction

Peak No.	Terminal Phosphorus[a] / Total Phosphorus[b]	Labile Phosphorus[c] / Total Phosphorus[b]	E_p, Optical Density[d]	5′-Nucleotide[e] / Nucleoside	Alkaline Hydrolysis[f]: Mononucleotide (% of total $O.D._{260}$ units)	Mononucleotide P[g] (% of total P)	Nucleoside di(tri) P[h] / Mononucleotide P[g]	Tentative Structure
1	0.91, 1.11	0	15,000		100	100		$_pX$ or X_p
2	0.77, 0.71, 0.63	0.36, 0.30, 0.25	7,200	0.8	68, 67, 64	33	1.96	$X_pX_p\sim p$
3	0.83, 0.83, 0.83	0.59, 0.56, 0.44	4,900	1.3	26, 22, 20	25	2.94	$X_pX_p\sim p\sim p$
4	0.31, 0.31, 0.32	0	10,600	2.8	92, 93, 82	93	0	$(X_p)_4$
5	0.22, 0.20, 0.22	0	9,400	4.0	85, 90, 95	94	0	$(X_p)_5$
6	0.32, 0.31, 0.28	0	7,500	4.4	87, 87, 90	77	3.70	$_p(X_p)_5$
Acid-insoluble RNA	0.025		7,900					

[a]Includes monoesterified phosphorus and pyrophosphate.
[b]Individual chromatographic separations and determinations.
[c]Adjusted to 1 *M* with respect to H_2SO_4 and heated at 100°C for 10 min.
[d]Optical density at 260 mμ per mole of phosphorus.
[e]Snake venom phosphodiesterase treatment after removal of terminal phosphorus by bacterial alkaline phosphatase.
[f]No nucleosides found in any of the hydrolysates.
[g]Nucleoside 2′- and 3′-phosphate.
[h]Includes nucleoside 3′,5′-diphosphate as in peak 6 and monoesterified phosphorus and pyrophosphate as in peaks 2 and 3.

and all the phosphorus of each fragment is converted to P_i upon treatment with bacterial alkaline phosphatase. The alkali-resistant fragment of peak 2 has a total phosphorus/labile phosphorus value of 2.1, whereas the corresponding fragment of peak 3 shows a value of 1.6. The phosphorus analyses and the difference in elution pattern from Dowex 1–chloride columns indicate that the alkali-resistant fragment of peak 2 is a nucleoside diphosphate, whereas the corresponding fragment of peak 3 is a nucleoside triphosphate. Since no nucleosides are liberated upon alkaline hydrolysis of peaks 2 and 3, the phosphate groups are most probably linked to C 2 or C 3 hydroxyl groups of the terminal nucleoside.

If peak 3 is indeed a dinucleotide terminating in a nucleoside triphosphate, then the net negative charge of these molecules will be the same as the tetranucleotides of peak 4. The ready chromatographic separation of these two peaks suggests that chain length or nucleotide composition, in addition to net charge, is contributing to the behavior of the molecules on DEAE cellulose.

Finally, Table 15.5.1 shows a terminal P/total P value of 0.025 for the acid-insoluble RNA recovered from DEAE-cellulose columns. This value indicates that the average molecular weight of acid-insoluble RNA is about 14,000.

[1]F. J. Finamore and E. Volkin, *J. Biol. Chem.* **236**, 443 (1961).

[2]F. J. Finamore, T. Yamada, and Rose P. Feldman, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 159–61.

[3]R. V. Tomlinson and G. M. Tener, *Biochemistry* **2**, 697 (1963).

15.6 INVESTIGATION OF NUCLEASES IN MAIZE

N. G. Sansing

Introduction. – After the third day of germination of maize seedling, a very high level of nucleases is attained. The roots and mesocotyl are particularly high in nuclease activity, making it very difficult to extract undegraded samples of rapidly labeled RNA. Some of the properties of these nucleases have been investigated using the filter-paper-disk assay previously described.[1]

Results and Discussion. – Chain length differences of hydrolytic products may be measured by comparing the retention of products on filter-paper disks washed in 5% trichloroacetic acid and on DEAE-cellulose disks washed with varying concentrations of NaCl and 1 *M* urea. The concentration of NaCl determines the length of oligonucleotide retained on the disk.[2] The interpretation of kinetic data using both filter-paper disks, which retain all oligonucleotides with a chain length greater than three nucleotides, and DEAE-cellulose disks, washed with a salt concentration which will retain only chain lengths greater than ten nucleotides, suggests that a major nuclease in corn roots is an exonuclease. The same kinetics of hydrolysis are found for the hydrolysis of labeled RNA and labeled, heat-denatured DNA, using a crude homogenate as an enzyme source. Native DNA, however, is degraded very slowly by the crude enzyme.

The glucosylated DNA of bacteriophage T2 is hydrolyzed by the maize enzyme, if heat denatured, although at a lower rate than is found with heat-denatured *Escherichia coli* DNA. Phage T2 DNA is resistant to many nucleases, and for this reason the maize nuclease may be of value as a preparative enzyme. The pH optimum for the reaction is approximately 6.0, and there is no demonstrable requirement for a metal ion as an activator.

Summary. – Efforts are currently under way to purify the nuclease which hydrolyzes heat-denatured T2 DNA so that the enzyme's properties and substrate specificities may be more carefully studied.

[1]N. G. Sansing, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 164–65.

[2]C. G. Mead, *Federation Proceedings*, in press.

15.7 ISOLATION, PURIFICATION, AND SOME PROPERTIES OF A NEW PHOSPHODIESTERASE FROM BRINE SHRIMP EGGS

A. H. Warner F. J. Finamore

Introduction. – The occurrence of a unique pyrophosphate ester, P^1P^4-diguanosine 5′-tetraphosphate (di-GDP), in *Artemia salina*[1] prompted

us to study the metabolism of this compound in the brine shrimp egg. In order to understand more fully the importance of di-GDP, it first became necessary to study an enzyme system capable of hydrolyzing di-GDP and to characterize the products resulting therefrom.

Method of Assay. – Since a bacterial alkaline phosphatase preparation (Worthington Co.) does not attack di-GDP but does release inorganic orthophosphate from GMP, GDP, and GTP,[1] we used an excess of alkaline phosphatase in our assay system. Consequently, any hydrolysis of di-GDP by enzyme present in the egg extracts could, under these conditions, be measured as release of P_i.

Isolation and Purification. – Dried brine shrimp eggs were ground with an electric mortar and pestle for 30 min and were extracted with cold 0.30 *M* NaCl containing 0.05 *M* Tris buffer, pH 7.4. The homogenate was centrifuged at 23,500 × *g* for 30 min, and the supernatant was decanted through several layers of cheesecloth.

To the straw-colored liquid was added solid $(NH_4)_2SO_4$ to 50% saturation. The insoluble material was removed by centrifugation and discarded. The bulk of the active material was then obtained at 50–100% saturation with $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in 0.10 *M* Tris, pH 8.5, and percolated through a DEAE-cellulose column. Contaminating nucleic acids were adsorbed to the cellulose, while virtually all the enzyme activity passed through the column.

The DEAE-treated protein fraction was adjusted to pH 4.2 with acetic acid, and the insoluble material was removed by centrifugation. The soluble fraction, containing about 80% of the original enzyme activity, was then adjusted to pH 7.4 with NaOH. The soluble fraction was next brought to 100% saturation with $(NH_4)_2SO_4$, and the protein precipitate was dissolved in a small volume of 0.30 *M* NaCl containing 0.025 *M* Tris, pH 8.5.

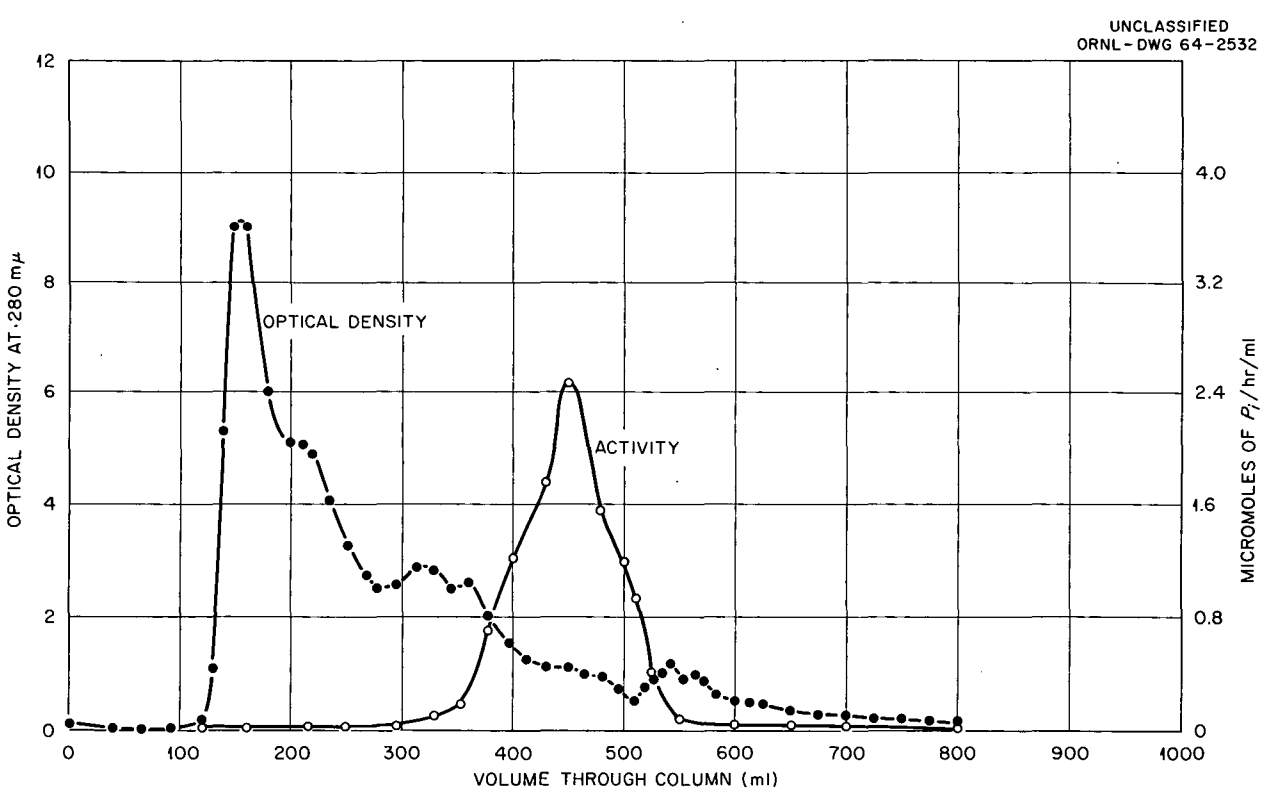

Fig. 15.7.1. Fractionation on Sephadex Column.

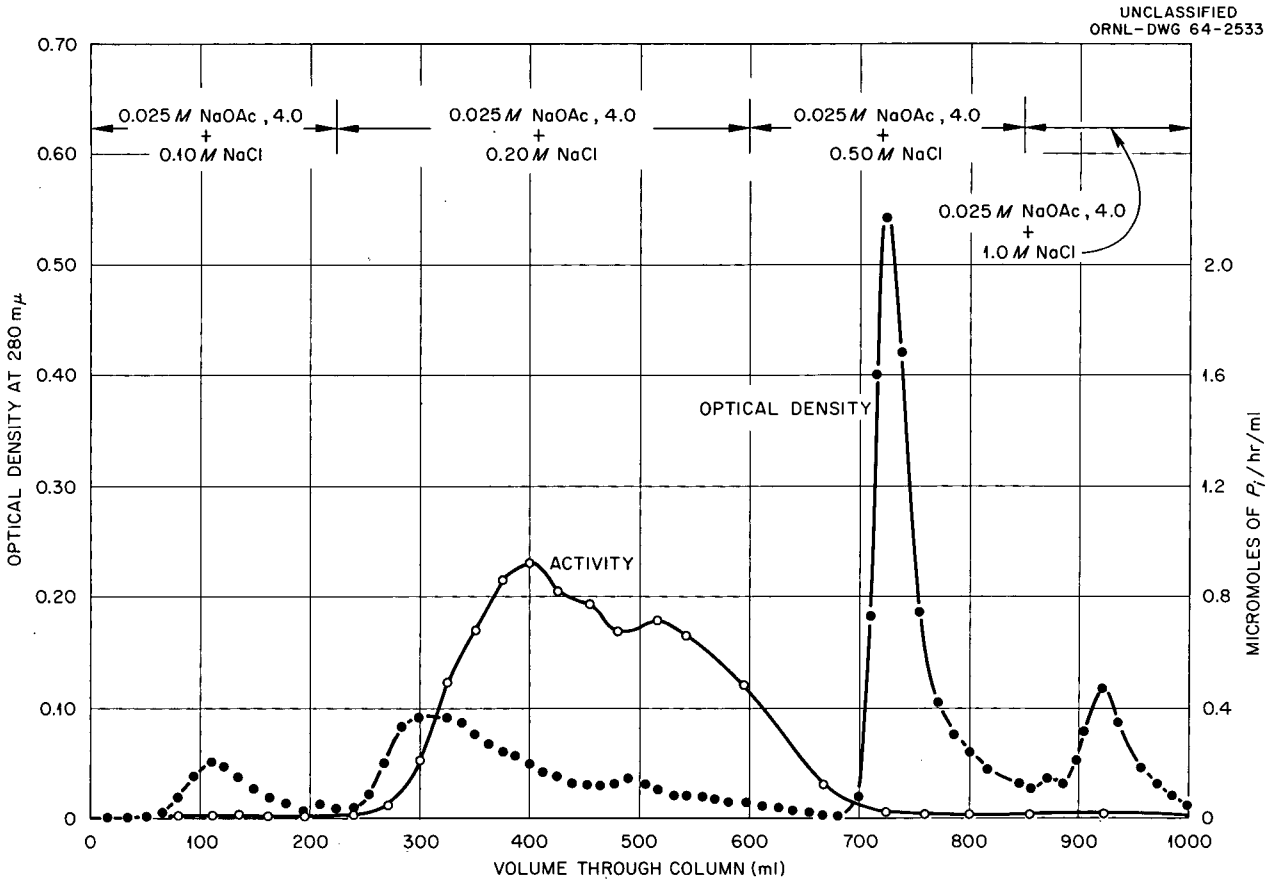

Fig. 15.7.2. Fractionation on Carboxymethyl Cellulose Column.

The concentrated protein was applied to a Sephadex G-100 column (3.5 × 70 cm), and filtration was carried out for 18 hr in the cold (Fig. 15.7.1). The contents of the tubes containing enzyme activity were pooled and dialyzed against 4 liters of 0.025 *M* Na-acetate, pH 4.0, for 2 to 3 hr. The dialyzed material was carefully adjusted to pH 4.0 with acetic acid and applied to a carboxymethylcellulose column (2.5 × 15 cm). The elution was carried out batchwise with increasing salt concentration (Fig. 15.7.2). The active material thus obtained was pooled, dialyzed against distilled water, and lyophilized. At this stage the enzyme was purified about 500-fold.

General Properties. – The enzyme fraction appears to have a pH optimum of 8 and to require Mg^{2+}. It is sensitive to chymotrypsin and relatively heat stable. Preliminary data obtained from the analytical ultracentrifuge indicate the enzyme to have a sedimentation constant of <1.

Specificity experiments suggest that the enzyme cleaves di-GDP asymmetrically into equimolar quantities of GMP and GTP and that it is inactive toward GMP, GDP, GTP, adenosine tetraphosphate, and inorganic sodium pyrophosphate.

[1]F. J. Finamore and A. H. Warner, *J. Biol. Chem.* **238**, 344 (1963).

15.8 STUDY ON VIRAL RNA POLYMERIZATION IN VIVO

R. K. Fujimura

Introduction and Method. – If RNA synthesis proceeds by sequential addition of mononucleotides, it may be possible to isolate RNA molecules

which are isotopically labeled over only a portion of their length. Such nonuniform labeling can be detected most readily if all the molecules are identical in base sequence and chain length. Bacteriophage R17 RNA is such a material.[1] Partially labeled R17 RNA was obtained by adding P^{32} to an R17 phage-infected *Escherichia coli* culture a short time before harvest. The infected cells were harvested and lysed. Only the phage particles of size similar to mature phage were isolated by the purification procedure, which involves sedimentation by high-speed centrifugation and banding by two cycles of sucrose density-gradient centrifugation. The preliminary studies have shown that partially labeled RNA may be obtained if P^{32} is added about 15 min or less before harvest of the infected cells. The total incubation period of infected cells was 80 min.

Viral RNA isolated from R17 phage was extensively hydrolyzed by pancreatic ribonuclease in the presence of a large excess of nonlabeled R17 RNA. If the specific activity ratio among the resulting polynucleotides is not constant, it should mean that the RNA was partially sequentially labeled.

Results and Discussion. – RNA isolated from phage obtained from cultures that incorporated P^{32} for the entire length of the culture period (sample I), for 40 min before harvest (sample II), and for 12 min before harvest (sample III) were treated with pancreatic ribonuclease extensively. The resulting polynucleotides in each sample were separated on the basis of chain length by means of a urea–DEAE-cellulose column using a sodium acetate gradient.[2] The distribution of absorbancy at 260 mμ is shown in Fig. 15.8.1, along with the statistically expected distribution. The distribution patterns agree very closely with the statistical distribution for all the samples, indicating that the degree of hydrolysis is quite reproducible. However, in every case about 2% of the polynucleotides did not come off until the addition of alkali. The specific activity ratio among the polynucleotides in each sample is shown in Fig. 15.8.2. It shows that RNA sample III is definitely nonuniformly labeled.

In addition to partial sequential labeling of RNA, there are two other obvious causes for a nonuniform specific activity ratio.

(1) Preferential Incorporation of P^{32} into Certain Mononucleotides More than Others. – A sample was prepared under almost the same conditions as those used in obtaining sample III. Polynucleotides from peak 3 were treated with alkaline phosphatase and then with snake venom phosphodiesterase, and after most of the inorganic P^{32} was removed, 5′-mononucleotides were isolated by a Dowex 1 formate column. The specific activity ratios among them were not constant. They were as follows, with respect to CMP = 1: AMP, 1.0; UMP, 1.7 (corrected for inorganic P^{32}); and GMP, 1.4. However, a calculation showed that these differences were not sufficient to cause such a marked difference in specific activity ratio of the polynucleotides. In Fig. 15.8.2 (sample IV) the result of such a calculation is shown. The base composition of polynucleotides in each chain length[3] was determined for sample II and was used for the calculation.

(2) Impurity. – Since the specific activity ratio for samples I and II came out essentially constant

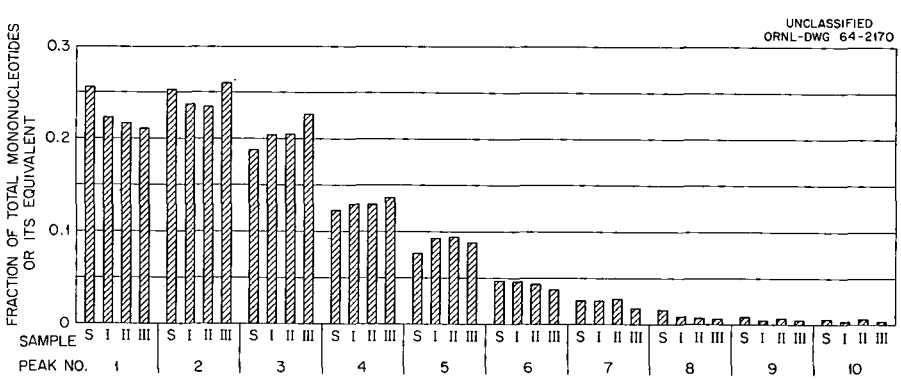

Fig. 15.8.1. Polynucleotide Distribution After Pancreatic Ribonuclease Digest.

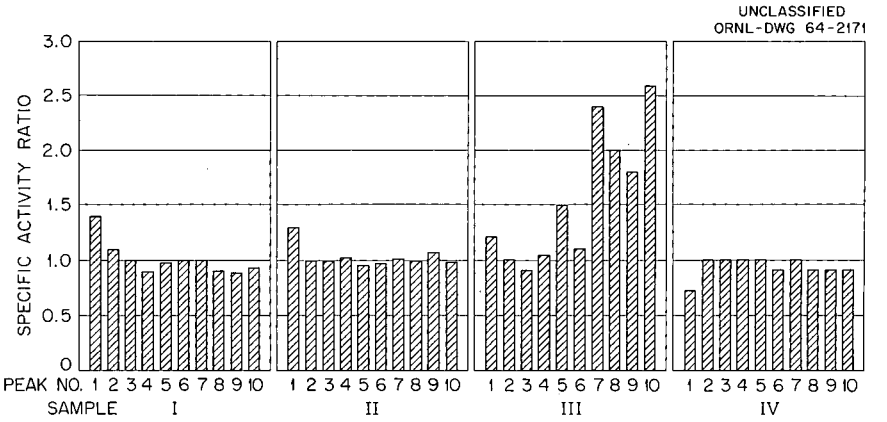

Fig. 15.8.2. Specific Activity Ratio Between Polynucleotides of Various Chain Lengths.

and ribosomal RNA synthesis is partially suppressed under these conditions,[4] the chance of contamination with non-R17 RNA in the short-time P^{32} incorporation sample is small. However, the possibility of impurity is still being investigated.

Thus the available data indicate that viral RNA is sequentially synthesized, and it is possible to isolate such an RNA. Attempts are being made to get a more definite answer by using an exonuclease hydrolysis.

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[1] S. Mitra *et al.*, *Proc. Natl. Acad. Sci. U.S.* **50**, 68 (1963).

[2] R. V. Tomlinson and G. M. Tener, *J. Am. Chem. Soc.* **84**, 2644 (1962).

[3] Base compositions were determined in collaboration with N. Sinha, Department of Biochemistry, University of Wisconsin.

[4] B. Ellis and W. Paranchych, *J. Cellular Comp. Physiol.* **62**, 207 (1963).

15.9 DESALTING OF NUCLEIC ACID AND ITS DERIVATIVES BY METAL HYDROXIDE PRECIPITATION

J. X. Khym

Introduction. – As a corollary to investigations of separating nucleic acid components by the techniques of reversed-phase partition chromatography, a procedure for desalting nucleic acid solutions is being examined. It was found that nucleic acids and lower-weight oligonucleotides are coprecipitated from sodium chloride or formate salt solutions when moderate amounts of added La^{3+} or Cd^{2+} are precipitated as their hydroxides from these same salt solutions.

Results and Discussion. – Removal of metal and recovery of the compounds coprecipitated are accomplished by various treatments of a water slurry of the precipitated hydroxides. The type of compounds that have been recovered from sodium chloride or sodium formate solutions and the manner of metal hydroxide removal are shown in Table 15.9.1. Recoveries after metal removal are about 90%.

Nucleic acid components at about 2×10^{-3} micromole/ml have been shown to be quantitatively precipitated. The total amount that can be precipitated depends upon the amount of carrier metal added (~25 micromoles of nucleoside phosphate are precipitated quantitatively with 0.1 millimole of lanthanum, and ~10 micromoles of nucleic acid with 0.1 millimole of cadmium). For convenience in recovery of precipitate it is necessary to use increasing amounts of carrier metal ions as larger volumes of salt are encountered (see Table 15.9.2).

Representative data showing the amount of salt that remains after carrier metal removal are shown in Table 15.9.2.

The completeness of removal of lanthanum and cadmium from the nucleic acid components is now being tested by radioactive tracer techniques. Also the extension of this coprecipitation technique for desalting nucleic acid solutions other than sodium formate or sodium chloride is being investigated.

Table 15.9.1. Removal of Metal Hydroxides

Carrier Metal	Treatment	pH After Removal	Used to Desalt
La	Add equivalent amount of $H_2C_2O_4$	2.0–2.3	Ribomononucleotides
La, Cd	Add strong acid exchanger, H-form	2.0–2.5	Negatively charged compounds insensitive to acid
La, Cd	Add weak acid exchanger, Na/H-form, pH 4.7	5.8–6.0	Nucleoside-PO_4, oligonucleotides, sRNA
Cd	Add strong acid exchanger, NH_4-form	8.0–9.0	RNA, DNA, sRNA

Table 15.9.2. Total Millimoles of Salt Present After Carrier Metal Removal

Initial NaCl Concentration	Initial Carrier Metal Added (millimoles)			
	0.1 La^{3+}	1.0 La^{3+}	0.3 Cd^{2+}	3.0 Cd^{2+}
	Volume (ml)			
	100	1000	100	1000
0.1 *M*	0.05	0.15	0.14	1.4
1.0 *M*	0.10	0.20	0.21	1.9
5.0 *M*	0.22	1.32	0.28	2.5

16. Chemical Protection and Enzyme Catalysis

D. G. Doherty[a]

J. D. Bales, Jr.
Margaret A. Turner

[a]Dual assignment.

16.1 CHEMICAL PROTECTION AND ENZYME CATALYSIS

D. G. Doherty J. D. Bales, Jr.
Margaret A. Turner

In view of the good protection afforded by mixtures of AET and glutathione (GSH),[1] it has seemed worth while to continue to explore the various parameters in the system in order to gain some insight as to the nature of its action. Previous evidence indicates that protection decreases rapidly as the time between administration and radiation is increased, presumably due to oxidation, metabolism, or excretion of the protective compound. If GSH is involved in the maintenance of sulfhydryl compounds in reduced form, then high concentrations of GSH should extend their effective period of action. Glutathione (1.6 g/kg intraperitoneally) given to $BC3F_1$ mice ($LD_{50/30}$, 720 r) 15 min prior to x radiation yields an $LD_{50/30}$ of 1050 r, while GSH when given 1 hr before radiation shows no protective effect. With AET (360 mg/kg intraperitoneally) the LD_{50} is 1500 r when the chemical is given 15 min before radiation and 1200 r when given 1 hr before radiation. The mixture of AET (480 mg/kg) and GSH (1.6 g/kg) intraperitoneally, with an initial LD_{50} of 1700 r at 15 min, drops to 1450 r at 1 hr, 1250 r at 2 hr, and 950 r at 3 hr. Thus, GSH effectively increases the span of action of AET administered intraperitoneally. The mixture also apparently protects the intestinal system better than the hemopoietic, as evidenced by the increased LD_{50} obtained when isologous bone marrow posttreatment is added to the chemical pretreatment. In both $1C3F_1$ and $BC3F_1$ mice, the injection of 50×10^6 fresh, isologous bone marrow cells one day after pretreatment with the mixture and x radiation raised the LD_{50} to approximately 2100 r – a dose reduction of nearly 3.

Investigation of the components of GSH for protective activity has so far shown them to have little activity. On a molar basis, cysteine is about one-half as active as GSH, 1.6 g/kg intraperitoneally yielding an LD_{50} of 1100 r. Cysteinylglycine and glutamylcysteine were synthesized and, on testing, at equal molar levels, found to have little or no protective effect. Apparently, the intact GSH molecule is necessary for reasonable protective activity.

Additional thiophosphate compounds have been prepared by procedures outlined in a previous report[2] and tested for protective activity. 2-Aminoethylthiophosphate monosodium salt at 480 mg/kg intraperitoneally gave an $LD_{50/30}$ of 1450 r, as did 1 g/kg of 3-aminopropylthiophosphate monosodium salt and 800 mg/kg of DL-2-aminopropylthiophosphate monosodium salt. These thiophosphate compounds are relatively stable at neutral pH, and it is unlikely that they are materially hydrolyzed to the aminomercaptan in the short time prior to irradiation.

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[1]D. G. Doherty, paper presented at the *Protection and Recovery Conference, Nov. 22–23, 1963, Oak Ridge* (abstract).

[2]D. G. Doherty, J. D. Bales, Jr., and M. A. Turner, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963,* ORNL-3427, p. 129.

17. Enzymology

G. D. Novelli[a]

Protein Synthesis (17.1–17.17)

G. D. Novelli
R. J. Mans
A. P. Pfuderer
Lawrence Rosen
M. P. Stulberg
C. J. Wust
Piero Cammarano[b]
D. P. Kosow[c]
Marialuisa Melli[b]
J. V. Schnell[d]
P. A. Swenson[e]
G. R. Williams[c]
Audrey N. Best[f]
D. R. Holladay
G. R. Holloway
Eunice L. Kincaid
Mary V. Long
R. D. MacElroy[g]
A. Marie Manning
Katherine H. McGrath

Hormonal Control (17.18–17.21)

F. T. Kenney
D. L. Greenman[h]
W. D. Wicks[c]

Mammalian Cell Regulation (17.22–17.23)

K. B. Jacobson[a]
J. B. Murphy[a]
P. J. Fritz[h]
T. H. Turney, Jr.[d]

Energy Metabolism (17.24–17.26)

H. D. Peck
J. T. Davidson
E. F. Phares
Sister M. Regina Lanigan[h]

Cooperative NIH-AEC Chemical Co-Carcinogenesis Program (17.1–17.3, 17.10–17.14, 17.18–17.21, 17.23)

[a]Dual assignment.
[b]Visiting investigator from abroad.
[c]American Cancer Society Fellow.
[d]Research associate.
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[f]Leave of absence, September 1963–September 1964.
[g]ORINS Fellow.
[h]USPHS Fellow.

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 attempts to improve the isolation procedure, we have prepared ribonuclease to study methods to separate it from the polymerase. The major use for the purified polymerase will be in the β-galactosidase system, where we hope to study the control mechanisms in protein synthesis at the level of the gene.

We have also continued to study the incorporation of amino acids in systems not involving ribosomes. These systems were discovered in this laboratory, and we are trying to assess the significance of such "soluble" systems in relation to the more conventional systems.

Our studies with the plant protein synthesizing systems have continued. This work is proceeding in two directions: (1) an attempt to understand the effect of preillumination of seedlings on the amino-acid-incorporating activity of the cell-free system and (2) an attempt to prepare a DNA-dependent RNA polymerase from maize in order to study the control of the biosynthesis of the gene-specific esterases.

As part of our effort in the Co-Carcinogenesis Program, we are studying the control of protein synthesis in regenerating liver and the control of nucleic acid synthesis in Novikoff hepatoma. We consider that studying control mechanisms in model system such as these and others like antibody synthesis and the induced β-galactosidase synthesis will reveal clues that will indicate which systems would be most advantageous to study during carcinogenesis.

We have undertaken a cooperative program with Chemical Technology to prepare sRNA from *E. coli* in large amounts in the hope that methods can be developed to separate the individual amino-acid-specific sRNA's in sufficient quantity to permit a detailed analysis of the nucleotide sequence in order to determine the nature of the code. In conjunction with this effort, it is necessary to have available amino-acid-activating enzymes in order to assay the sRNA's. Also we are trying to purify some of the activating enzymes in sufficient purity and quantity that will permit the study of the manner by which such enzymes recognize the correct species of sRNA. All these activities, as well as others detailed in this report, depend for their success on the pilot-plant operation. In addition to our needs, we have provided other groups in the Division with cells or other materials (see Chap. 17.17).

17.1 CONTINUATION OF STUDIES ON THE PURIFICATION OF THE DNA-DEPENDENT RNA POLYMERASE FROM *ESCHERICHIA COLI*[1]

A. Marie Manning Mary V. Long
G. D. Novelli

Introduction. – This is a continuing investigation of the large-scale purification of the DNA-dependent RNA polymerase from *E. coli* that will be used in the cell-free synthesis of a number of specific proteins.

Results and Discussion. – In the previous report[2] we described a procedure that gave us a 100-fold purification of the enzyme with a 60% yield. At this stage the enzyme is stable when stored in liquid nitrogen. During the current period, we processed almost 6 kg of wet cells to give 6.7×10^6 enzyme units or about 1.2 g of enzyme, if it were completely pure. We are currently trying to devise a column method for the further purification of the enzyme. The DEAE column procedure of Chamberlin and Berg[3] does not work in our hands. Our results, since the bulk of the enzyme comes off in the "breakthrough," suggest that the column does not have adequate capacity under the conditions we employ. Since our initial steps are quite different from those of Chamberlin and Berg, it is possible that we may have materials in our preparation that have a greater affinity for the column than our enzyme. Therefore we are exploring the use of other types of columns.

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[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]L. Rosen, A. Marie Manning, and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 176.

[3]M. Chamberlin and P. Berg, *Proc. Natl. Acad. Sci. U.S.* **48**, 81 (1962).

17.2 ISOLATION OF RIBONUCLEASE FROM *ESCHERICHIA COLI* RIBOSOMES[1]

Mary V. Long G. D. Novelli

Introduction. – In the purification of the DNA-dependent RNA polymerase,[2] the step that limits the amount of material that can be worked up at one time is a centrifugation at $105,000 \times g$ to remove ribosomes. The ribosomes must be removed to get rid of RNase, which would hydrolyze the product of the RNA-polymerase reaction. If the Spinco centrifugation could be avoided, about ten times as much material could be worked up at one time. We found previously that the ribosomes can be removed by precipitation with $MnCl_2$, but this process liberates some RNase. If, however, the RNase could be removed from the RNA polymerase

Table 17.2.1. Ribonuclease from Lac⁻ *E. Coli* Ribosomes

	Column Load (mg)	Protein (mg)	Ribonuclease Activity (relative units)	Activity per Milligram of Protein
Ribosome solution		18,000	5,040	0.28
Urea supernatant		2,830	26,600	9.4
$(NH_4)_2SO_4$ fraction 50–80%		630	13,000	20.7
CG-50 Amberlite column	70	2.4	338	141
Sephadex G-75 column	60	24.6	1,940	79
	170	43.7	4,022	92
Carboxymethyl-cellulose column	12	2.2	826	375
	43.7	2.2	1,090	495
		5.0	1,092	218

at a later stage, we could utilize the $MnCl_2$ precipitation step. We therefore have purified the ribosomal RNase in order to mix it with our RNA polymerase and attempt to separate the two enzymes on a column.

Results and Discussion. – Starting with approximately 800 g of *E. coli* cells, we obtained about 70 mg of ribosomes. These were used to purify ribonuclease by a modification of the procedure of Spahr and Hollingworth.[3] Greater purification was obtained by chromatography of the ammonium sulfate fraction on Sephadex G-75 or carboxymethyl-cellulose columns adapted from the procedure of Hartley *et al.*[4] A summary of the purification is given in Table 17.2.1.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]L. Rosen, A. M. Manning, and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 176.

[3]P. F. Spahr and B. R. Hollingworth, *J. Biol. Chem.* **236**, 823 (1961).

[4]R. W. Hartley, Jr., *et al.*, *Biochemistry* **2**, 794 (1963).

17.3 STUDIES ON THE CELL-FREE SYNTHESIS OF β-GALACTOSIDASE[1]

Lawrence Rosen G. D. Novelli

Introduction. – Attempts have been made again to obtain the cell-free system from preinduced *Escherichia coli* B which has the ability to synthesize β-galactosidase *de novo*.[2,3] Such cell-free synthesis is dependent upon the presence of inducer and could only be prepared when the cells had been broken in a French pressure cell. One of the major reasons for again obtaining and studying the above system is to determine the role of the inducer in induced protein synthesis.

Current reports indicate that polysomes are important for protein synthesis in cell-free systems derived from *E. coli*.[4,5] Therefore, different breaking pressures were used in the French pressure cell to rupture *E. coli* B cells in order to see if the yield of polysomes was affected by the breaking pressure. The yield and characteristics of the ribosomes as a function of the breaking pressure were then determined by the use of sucrose density gradients which fractionate ribosomes by molecular weight.

Results and Discussion. – Breaking pressures of 8000 to 20,000 psi were used, and the broken-cell preparation was then fractionated to obtain the components necessary for the cell-free synthesis of β-galactosidase, a high-speed supernatant (S_{90}) and a ribosomal fraction. The ribosomal fraction was prepared by either the Wettstein procedure, in which the ribosomes are washed by centrifugation through sucrose layers[6] (P_{90W}), or by the procedure previously used[2] (P_{90}). As the breaking pressure was increased, greater amounts of both S_{90} and P_{90} protein were obtained per unit weight of cells. Typical yields of P_{90W} were 1.1 and 2.2 mg of ribosomal protein per gram of wet weight of cells at 8000 and 19,000 psi respectively.

The patterns obtained from these ribosomes (P_{90W}) by centrifugation through sucrose density gradients (10–34% w/v) were similar; the only OD_{260} peak observed was in the 70S region, although slight absorbancy, without peaking, was observed in the heavier region. Assay of these fractions for β-galactosidase content indicated peaks of enzyme activity up to 143S (nominal).

Attempts to synthesize β-galactosidase with these cell-free fractions have been generally unsuccessful, although increases in enzyme activity during the reaction have been obtained occasionally, but no absolute dependence on added inducer could be demonstrated.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]J. M. Eisenstadt, T. Kameyama, and G. D. Novelli, *Proc. Natl. Acad. Sci. U.S.* **48**, 652 (1962).

[3]T. Kameyama and G. D. Novelli, *Proc. Natl. Acad. Sci. U.S.* **48**, 659 (1962).

[4]W. Gilbert, *J. Mol. Biol.* **6**, 374 (1963).

[5]T. Staehelin *et al.*, *Nature* **199**, 865 (1963).

[6]F. O. Wettstein, T. Staehelin, and H. Noll, *Nature* **197**, 430 (1963).

17.4 AMINO ACID INCORPORATION BY EXTRACTS OF *ESCHERICHIA COLI*

J. V. Schnell G. D. Novelli

Introduction. – Recent investigations point to the existence of at least three peptide-bond-synthesizing pathways catalyzed by bacterial extracts. These are: the ribosome-dependent synthesis of proteins, the "soluble amino-acid-incorporating system,"[1] and the polypeptide synthetases.[2] The role of each of these mechanisms to the overall biosynthesis of proteins remains to be established.

Previously, Stulberg *et al.*[3] had described an amino-acid-incorporating system from extracts of *Escherichia coli* which had several interesting features. With these preparations 75% of the labeled *L*-leucine incorporated into hot trichloroacetic acid–insoluble material was free of the ribosomes. This observation is in contrast to the more common result in which about 90% of the incorporated radioactivity is bound to the ribosomes. In addition, this system was stimulated by an unidentified factor present in several DNA preparations tested.

The system just described is being reinvestigated for several reasons. The occurrence of large amounts of the radioactivity in the soluble form suggests that this system might be useful for a study of the mechanism of the release of proteins from the ribosomes. It is also possible that a detailed study of the distribution of incorporated radioactivity will provide useful insights into the contribution of each of the three known peptide-synthesizing mechanisms to the overall incorporation observed.

Results and Discussion. – An attempt to reproduce the system of Stulberg *et al.* has resulted in supernatant and ribosomal preparations which incorporate labeled L-leucine with a 50% recovery of the radioactivity in the soluble form. Titration of the supernatant fraction with protamine sulfate increased the dependency of the incorporation on added transfer sRNA and also removed about 85% of the "soluble" amino-acid-incorporating activity of the supernatant fractions. Washing of the ribosomal fraction by repeated ultracentrifugation reduced nonsupernatant-dependent incorporation substantially. Experiments are presently in progress to investigate the process of the release of proteins from the ribosomes.

[1]A. Kaji and G. D. Novelli, *Biochem. Biophys. Res. Commun.* **10**, 406 (1963).

[2]Mirko Beljanski and Monique Beljanski, *Biochim. Biophys. Acta* **72**, 585 (1963).

[3]M. P. Stulberg *et al.*, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1961*, ORNL-3201, p. 137.

17.5 INHIBITION OF MACROMOLECULAR SYNTHESIS IN *ESCHERICHIA COLI* BY ULTRAVIOLET IRRADIATION

P. A. Swenson R. B. Setlow[1]

Introduction. – Ultraviolet doses of 5 ergs/mm^2 at 2650 A completely block DNA synthesis in *Escherichia coli* B_{s-1}, a radiation-sensitive strain.[2] Irradiation of strains B and B/r also blocks their DNA synthesis, but the bacteria recover from ultraviolet doses of 200 ergs/mm^2 and resume synthesis at normal rates.

Results and Discussion. – We have compared the effects of irradiation at 2650 A on the incorporation of H^3-thymidine, H^3-uridine, and H^3-leucine in these strains. Incorporation of label was taken as a measure of DNA, RNA, or protein synthesis, all of which increase exponentially in unirradiated cultures. In the three strains the rate of RNA synthesis decreases with increasing dose, and the synthesis rate is constant during the time that DNA synthesis is blocked. Protein synthesis parallels RNA synthesis in irradiated B and B/r; in B_{s-1}, protein synthesis is affected slightly less than RNA synthesis and its rate increases slowly while DNA synthesis is blocked. When DNA synthesis resumes in B and B/r, RNA and protein synthesis rates increase exponentially. We conclude that the amount of RNA and protein synthesized per unit time in growing cells is directly proportional to the number of DNA units serving as templates for RNA production.

[1]Biophysics Group.

[2]R. B. Setlow, P. A. Swenson, and W. L. Carrier, *Science*, in press.

17.6 AMINO ACID INCORPORATION BY CELL-FREE EXTRACTS OF *CLOSTRIDIUM PASTEURIANUM*

R. D. MacElroy H. D. Peck, Jr.
G. D. Novelli

Introduction. – Studies of amino acid incorporation by cell-free extracts of *Clostridium pasteurianum* are continuing in an attempt to obtain the biosynthesis of ferredoxin in a cell-free system. In addition to the amino-acid-incorporating system requiring ribosomes and soluble protein reported previously,[1] the possible role of a soluble amino-acid-incorporating system in the synthesis of ferredoxin is being investigated.

Results and Discussion. – Extracts are prepared from frozen cells by means of the French pressure cell, and intact cells and cellular debris are removed by centrifuging first at 15,000 × *g* for 1 hr and then at 30,000 × *g* for the same time. A ribosomal pellet is obtained by centrifuging at 78,000 × *g* for 2.5 hr. Further centrifugation at 144,000 × *g* for 2 hr yields a second smaller ribosomal pellet and soluble protein essentially free of ribosomes. After dialysis for 24 to 36 hr, this soluble preparation incorporates leucine and phenylalanine (of 15 amino acids tested) into material that is not soluble in hot trichloroacetic acid. In sucrose density gradients the incorporated radioactivity appears to be associated with the soluble protein.

The incorporation of leucine and phenylalanine by this soluble system is dependent upon ATP and Mn^{2+} and is stimulated by Mg^{2+}, glutathione, and an energy-generation system. Although pancreatic ribonuclease completely inhibits amino acid incorporation, the addition of polyuridylic acid to the system does not stimulate the incorporation of phenylalanine. As this soluble incorporating system is not inhibited by chloramphenicol, it appears to be very similar to that extracted from *Escherichia coli* by Kaji *et al.*[2]

[1]R. D. MacElroy, H. D. Peck, Jr., and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 175.

[2]A. Kaji, H. Kaji, and G. D. Novelli, *Biochem. Biophys. Res. Commun.* 10, 406 (1963).

17.7 SOLUBLE AMINO-ACID-INCORPORATING SYSTEM FROM RAT LIVER

D. P. Kosow G. D. Novelli

Introduction. – A previous report from this laboratory has described a soluble extract from rat liver which incorporates C^{14}-arginine into a protein fraction.[1] Since the original isolation of

this amino-acid-incorporating system was based on a similar system from another species, the isolation and properties of the system were reinvestigated.

Results and Discussion. – Rat liver homogenates were freed of microsomes by high-speed centrifugation. This supernatant fraction, when supplemented with ATP, Mg^{2+}, and rat liver sRNA, is able to incorporate 0.03 micromole of C^{14}-arginine per mg of protein into a trichloroacetic acid–insoluble product. The supernatant fraction has been partially resolved into what appear to be three components by $(NH_4)_2SO_4$ fractionation. The amino-acid-incorporating system is highly sensitive to changes in ionic strength, which makes further purification of the components difficult. This system is similar to the classical amino-acid-incorporating systems in that it requires sRNA and ATP; however, it differs from the classical systems in that there is neither a requirement for a particulate fraction nor for GTP.

Work is continuing on the nature of the three components and the product(s) of the reaction.

[1]H. Kaji, G. D. Novelli, and A. Kaji, *Biochim. Biophys. Acta* **76**, 474 (1963).

17.8 STUDIES ON THE EFFECTS OF LIGHT ON PROTEIN SYNTHESIS IN PLANTS

G. R. Williams G. D. Novelli

Introduction. – Effects of preillumination on the activity of a subsequently prepared cell-free amino-acid-incorporating system from etiolated maize seedlings[1] have been studied further. With an altered isolation procedure we have obtained more active ribosome preparations, particularly from seedlings older than two days. This modification has permitted us to examine the light effect as a function of seedling age. Dose requirements have been investigated with respect to light intensity, light quality, and duration of exposure. Also the development and decay of the response have been examined.

Results and Discussion. – Although the total incorporating activity of ribosome preparations from five-day-old seedlings is only 25 to 35% of that obtained using two-day seedlings, it is more consistent and the relative magnitude of the light response is somewhat higher in the older seedlings. Thus five-day-old seedlings have been used in all subsequent experiments.

Routinely, a 60 to 70% stimulation of incorporating activity of ribosomes from five-day-old seedlings is obtained after the plants are exposed to high-intensity white light for 3 hr. A similar response is found when red light (100 $\mu w/cm^2$) is administered for 30 min followed by a dark period of 150 min. The magnitude of this response is unaffected by doubling the light intensity or by extending the period of light exposure to 3 hr. However, no response to red light is observed when the intensity of a 30-min treatment is reduced to 20 $\mu w/cm^2$. Treatments consisting of 30 min light plus 150 min dark result in no stimulation of cell-free amino acid incorporation when green light (104 $\mu w/cm^2$) is used, and very little effect is noted when blue light (106 $\mu w/cm^2$) is employed.

After a treatment period of 2 hr with red or white light, an additional period of 1 to 2 hr (in either light or dark) is required for the observed stimulation to develop. If the interval between light treatment and ribosome preparation is extended to 12 hr, the observed stimulation is slightly reduced; and after an intervening period of 40 hr, no effect on the incorporating system is observed.

Additional trials of the effect of preillumination on the level of polysomes have confirmed and extended the preliminary results reported previously.[2] A small, consistent increase in the level of heavy particles observed after sucrose gradient centrifugation is obtained in preparations from preilluminated maize shoots. Gradient analyses of similar ribosome preparations after a brief incubation in a reaction mixture containing radioactive amino acids indicate that the increase in amino acid incorporation as a result of light pretreatment is associated primarily with the heavier particles.

The observations regarding the magnitude and quality of light eliciting the response and the duration of the response are consistent with the hypothesis that the phenomenon under investigation is an early manifestation of the light-induced developmental changes observed in intact plants.

Investigation of the effects of light on amino acid incorporation using albino mutants to further confirm this speculation are contemplated.

Studies concerning the effect of light on the synthesis of messenger RNA are being initiated in an effort to clarify the relationship between the perception of light and the stimulation of protein synthesis. In addition, experiments will be undertaken to localize the intracellular site of light perception and the respective roles (if any) of nuclear and chloroplast DNA in this process.

[1]G. R. Williams, R. J. Mans, and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 182.

[2]G. R. Williams, R. J. Mans, and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 183.

17.9 ISOLATION OF A DNA-DEPENDENT RIBONUCLEOTIDE INCORPORATING ACTIVITY FROM MAIZE SEEDLINGS

R. J. Mans

Introduction. – The biosynthesis of a specific protein by isolated ribosomes can be directed by inclusion of the product of DNA-dependent RNA polymerase (messenger or informational RNA) in an appropriate incubation mixture. In pursuing the cell-free synthesis of the genetically characterized pH 7.5 esterase in maize, an enzyme preparation requiring DNA for ribonucleotide incorporation has been isolated from maize seedlings. Preliminary results on the isolation and properties of this activity are reported here.

Results and Discussion. – Previously, a chromatin fraction capable of incorporating labeled ribonucleotides into acid-precipitable material has been isolated from maize seedlings.[1] However, this preparation does not respond to added DNA. It was noted that the bulk of the ATP-incorporating activity of a crude homogenate is in the 4000 × *g* supernatant after sedimentation of 90% of the cellular DNA as chromatin. A portion of the supernatant ATP-incorporating activity requires the addition of the other three ribonucleoside triphosphates (XTP's).

Further centrifugation of the ATP-incorporating activity revealed that 90% of the XTP-requiring activity was in the 20,000 × *g* supernatant. The addition of calf thymus DNA (a generous gift from F. T. Kenney) stimulated ATP incorporation tenfold. UTP incorporation was also found to be DNA and XTP dependent, indicating that the reaction probably involves incorporation of all four RNA precursors. Inclusion of DNase, RNase, actinomycin D, and inorganic phosphate in the incubation mixture completely inhibits the DNA-dependent ribonucleotide incorporation. The addition of Mg^{2+} or Mn^{2+} as well as high ionic strength is required for maximum incorporation. The reaction is linear with time for 40 min, and the rate of ATP incorporation is linear with protein concentration. Although all these properties are analogous to those described for DNA-dependent RNA polymerase isolated from other sources, identification of the activity found in maize requires the isolation and characterization of the product. The low level of activity (20 micromicromoles of ATP incorporated per minute per milligram of protein) of this crude preparation is not adequate for isolation of the product.

Large-scale preparations of the crude homogenate of seedling material from 50-lb batches of seed are active and are relatively stable to storage at 0°, freezing and thawing, and prolonged storage at −20°. Preliminary purification of this material revealed that activity is independent of ribosomes and can be purified about threefold by ammonium sulfate precipitation. The nucleotide incorporation catalyzed by this preparation is completely dependent on DNA as well as the presence of all four XTP's for activity. DNA isolated from maize endosperm will substitute for the thymus DNA in this reaction.

[1]R. J. Mans, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 136.

17.10 CONTROL OF PROTEIN SYNTHESIS DURING LIVER REGENERATION[1]

Piero Cammarano Marialuisa Melli
G. D. Novelli

Introduction. – As mentioned in a previous report,[2] evidence has been presented that shows

an enhancement of protein synthesis in rat liver following a partial hepatectomy.[3] The present investigation is concerned with a study of factors regulating this enhanced protein synthesis.

Results and Discussion. – In vitro amino acid incorporation has been studied using both ribosomal and microsomal systems prepared at various times following a partial hepatectomy. When ribosomal systems were used, the ribosomes obtained at various times during regeneration were tested for amino-acid-incorporating activity in the presence of their homologous supernatants and compared with a ribosomal system from resting liver. Whenever differences were observed, the ribosomes from regenerating liver were tested with supernatant from normal liver and vice versa to ascertain which component was responsible for the increased incorporating activity. These experiments showed that the increased incorporating activity was caused by a component of the supernatant that could be observed at 24 hr following hepatectomy. Ribosomes from 24-hr regenerating liver have the same incorporating activity as normal ribosomes when tested with normal supernatant. On the other hand, normal ribosomes are stimulated 60 to 100% by the 24-hr supernatant. Microsomes from regenerating liver, when tested with either normal or regenerating liver supernatant, show an increasingly higher incorporating activity as a function of time after hepatectomy up to 24 hr. Microsomes obtained 48 hr after hepatectomy are about 50% as active as those obtained at 24 hr, but they are still twice as active as normal microsomes. In contrast to the ribosomal system, normal microsomes are not stimulated by the 24-hr supernatant. On the other hand, 24-hr microsomes lose very little of the incorporating activity when the homologous supernatant is replaced by a normal supernatant. By use of polyuridylic acid as a synthetic messenger, similar studies with ribosomal and microsomal systems were carried out. The polyuridylic acid system proved to be a more sensitive indicator of the changes taking place during liver regeneration. It can be concluded that the increase in protein synthesis following a partial hepatectomy is a consequence of the progressive removal of an inhibitory component from the microsomes and the gradual accumulation of a stimulatory component in the supernatant.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]G. Giudice, P. Cammarano, and G. D. Novelli, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 177.

[3]T. Hultin and A. Von Der Decken, *Exptl. Cell Res.* 13, 83 (1957).

17.11 STUDIES ON NUCLEIC ACID SYNTHESIS WITH NOVIKOFF ASCITES TUMOR[1]

Marialuisa Melli G. D. Novelli

Introduction. – This investigation, which has only recently begun, represents an attempt to study nucleic acid synthesis in extracts from nucleoli from the Novikoff hepatoma.

Results and Discussion. – The Novikoff ascites tumor is carried in 180- to 200-g female Sprague-Dawley rats and harvested for experiments on the sixth to seventh day. The cells are removed from the ascites fluid by centrifugation for 5 min at 5000 rpm in the Servall centrifuge. The tumor is quite hemorrhagic, but the red cells, although they sediment with the tumor cells, are lysed during sonication. To obtain nuclei, the tumor cells are suspended in isotonic sucrose (0.25 *M* sucrose – 0.005 *M* $CaCl_2$) and sonicated for 50 sec in a Raytheon 10-kc sonic oscillator. When nucleoli are to be obtained, the cells are sonicated for 1 min. Nuclei are collected by centrifugation for 10 min at 600 × *g* in the International centrifuge. The pellet is suspended in 1 *M* sucrose and layered on top of an equal volume of a 2 *M* sucrose solution and centrifuged for 30 min at 40,000 rpm in the Spinco centrifuge.

Nucleoli are obtained by centrifugation for 5 min at 1000 × *g* in the International centrifuge. The pellet is suspended in 0.5 *M* sucrose (0.05 *M* sucrose – 0.003 *M* $CaCl_2$) and layered on top of a 0.9 *M* sucrose solution and centrifuged for 20 min at 40,000 rpm in the Spinco centrifuge.

The purity of the preparations is controlled by examination with the light microscope and with the phase contrast microscope. The isolated nucleoli will be tested for their ability to catalyze the incorporation of ribonucleotide triphosphates into RNA. The base ratio of the newly synthesized RNA will be determined to see if it is ribosomal RNA precursor.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

17.12 CONFIGURATION STUDIES ON RAT LIVER POLYSOMES IN SOLUTION[1]

A. P. Pfuderer D. R. Holladay
Piero Cammarano G. D. Novelli

Introduction. – Electron micrographs of polysomes disclose two different configurations, the open and the clustered.[2] It has been thought that the clustering could be caused by air drying the sample for electron microscopy.

The sedimentation constants of rat liver polysomes were therefore determined at infinite dilution in the analytical ultracentrifuge to see if the frictional coefficients determined from them could be used to distinguish between these two configurations. These sedimentation coefficients were then used to calibrate sucrose density gradient centrifuge runs in order to obtain information on the higher polysomes.

Results and Discussion. – The sedimentation coefficients determined in this way are listed in Table 17.12.1. Frictional ratios were determined from them by the formula

$$\frac{f}{fo} = \frac{83n^{2/3}}{S_{20,w}},$$

assuming that the $n = 1$ polysome is a sphere with unit frictional ratio.

If we separate the frictional ratio into $f/fo = (f/fe)\cdot(fe/fo)$, where the first factor involves hydration and the second asymmetry, then the frictional ratio determined above is that caused by asymmetry alone, assuming that all the polysomes have the same hydration as the first. The axial ratios of the equivalent ellipsoid determined from the frictional ratio has been plotted vs n, the number of ribosome units, in Fig. 17.12.1.

The axial ratio is seen to increase rapidly for the first three polysomes and then to level off at a constant slope. The axial ratio for $n = 2$ is exactly that to be expected of two 230-A spheres rigidly held 100 A apart. The axial ratio for $n = 3$ falls short of a linear model. Using the same ribosome and spacing dimensions, one finds that the third ribosome would have to go in at an angle of approximately 90°. If the fourth also goes in at a 90° angle, one forms either a square or the start of a helix. The slope of the line from $n = 3$ to $n = 12$ can be used to determine the pitch of an

Table 17.12.1. Sedimentation Coefficients of Polysomes

n	$S_{20,w}$ Analytical Runs	$S_{20,w}$ Sucrose Gradient Runs[a]
1	83 ± 3 S	85 ± 1 S
	105 ± 2 S	
2	123 ± 4 S	122 ± 3 S
3	154 ± 4 S	156 ± 5 S
4	183 ± 5 S	180 ± 5 S
5	211 ± 10 S	211 ± 4 S
6		237 ± 3 S
7		257 ± 6 S
8		(282) S
9		299 ± 5 S
10		316 ± 6 S
11		
12		(353) S

[a]Values in parentheses were only observed once.

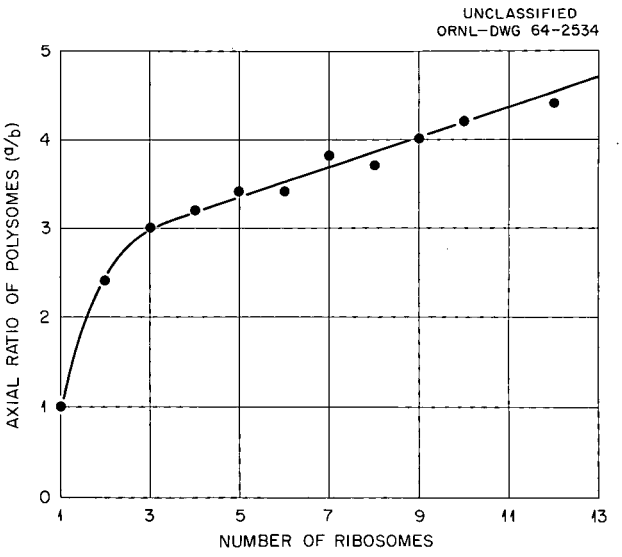

Fig. 17.12.1. Experimental Axial Ratio of Polysomes vs Number of Ribosome Units.

assumed helix by the formula

$$\tan (90° - \phi) \cong \frac{d(a/b)}{dn} \frac{(2L + D)}{(L + D)},$$

where ϕ is the pitch of the helix, L is the spacing between the ribosomes, and D is the diameter of the ribosome. Using the previous dimensions, one obtains a pitch of 73°.

Behnke[3] and Waddington and Perry[4] have observed polysome helices with a pitch of 60 to 70° in thin-section electron micrographs of embryonic tissue. Using 230 A as the ribosome diameter, 100 A as the spacing between ribosomes, and 70° as the helix pitch, one obtains a structure very similar to their electron micrographs.

The properties of rat liver polysomes in solution are those of a clustered, rather than an open, type of configuration. The data are very suggestive of the formation of the type of helix seen by Behnke and others in embryonic tissue. The frictional coefficient of the $n = 2$ polysome suggests that it is held together by a rigid link, with none of the characteristics of single-stranded RNA.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]J. R. Warner, A. Rich, and C. E. Hall, *Science* **138**, 1399 (1962).

[3]O. Behnke, *Exptl. Cell Res.* **30**, 597 (1963).

[4]C. H. Waddington and M. M. Perry, *Exptl. Cell Res.* **30**, 599 (1963).

17.13 EFFECT OF ACTINOMYCIN D ON THE IMMUNE RESPONSE[1]

C. J. Wust

Introduction. – It has been reported[2] that actinomycin D administered to rats following a second injection of β-galactosidase as antigen resulted in a depression in the titer of circulating antibody observed at nine days. A further evaluation of the effect of the drug on the immune response to a second antigen, sheep erythrocytes, has been made.

Results and Discussion. – Sprague-Dawley rats given a single intraperitoneal (ip) injection of 1 ml of a 5% suspension of sheep erythrocytes elicit maximally high hemagglutinin titers within five days. The titer rises after one day with an average titer of 3 to 4 $\log_2$ units at two days. When actinomycin D was injected simultaneously with the antigen, there was a delay in the appearance of circulating antibody, but there was no effect on the rate of the maximum amount of hemagglutinin produced. The delay was roughly proportional to the concentration of the antibiotic administered, up to two days for 75 μg in a 200-g female rat. The highest dose of actinomycin (75 μg) used had no effect on the animals in terms of the toxic manifestations described by Philips *et al.*[3] although this was the 50% lethal dose for 200-g male rats within two days with the syndrome reported by these authors.

The dose of 75 μg of actinomycin was given at various intervals prior to and following the ip injection of sheep erythrocytes. The greatest delay in the appearance of hemagglutinin (36 hr) occurred when the drug was given 8 hr before the antigen. There was a decreasing effect when the drug was given subsequent to the antigen, so that a minimal influence was observed if the antibiotic was given 6 hr before the antigen. Minimal or no effect was observed when the drug was given 20 hr before the antigen.

The effect of actinomycin was studied further in the mouse. In four-month-old LAF_1 mice, 10 μg of the antibiotic given ip 6 to 8 hr before an intravenous (iv) injection of 1 ml of a 10% suspension of sheep erythrocytes resulted in a 24-hr delay in the appearance of circulating hemagglutinin. There was no effect if the actinomycin was given at any other time or if the antibiotic was given iv 7 hr before the antigen.

The effect of actinomycin suggests that a synthesis of a specific messenger RNA may be required prior to the synthesis of antibody since this drug has been shown to affect nucleic acid metabolism in vivo and inhibit DNA-dependent RNA polymerase reactions in vitro.[4] This synthesis appears to occur within 6 hr after the injection of antigen. Studies in conjunction with

M. G. Hanna of the Mammalian Recovery Group are now in progress to determine the histological changes occurring with the antibiotic in the mouse.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]C. J. Wust and Clara L. Gall, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963,* ORNL-3498, pp. 180–81.

[3]F. S. Philips *et al., Ann. N.Y. Acad. Sci.* **89**, 348 (1960).

[4]J. Hurwitz and J. T. August, vol. 1, p. 59 in *Progress in Nucleic Acid Research* (ed. by J. N. Davidson and W. E. Cohn), Academic Press, New York, 1963.

17.14 CELL-FREE SYNTHESIS OF SHEEP ERYTHROCYTE HEMAGGLUTININ[1]

C. J. Wust

Introduction. – The cell-free incorporating system previously described[2] was studied for its ability to synthesize hemagglutinin to sheep erythrocytes. This antigen, in contrast to enzyme antigens used in former studies, elicits the production of serum antibody within two days in the rat following an intraperitoneal injection. This short period of time enabled us to evaluate the early sequences of synthesis before the appearance of serum antibody. The information that antigen transmits to the immunologically competent cell must be given very shortly after injection,[3] and it is the overall purpose of these studies to establish the role of antigen in inducing an immune response.

Results and Discussion. – The cell-free incorporating system has been improved by the following modifications:

1. Washed bentonite (1 mg/ml) is added to the homogenization medium.
2. Upon isolation of ribosomes at 105,000 × *g* for 90 min, the pellet is suspended in medium A and centrifuged at 30,000 × *g* for 20 min; the supernatant is centrifuged at 105,000 × *g* for 60 min, and the ribosomes isolated in this centrifugation are stored in liquid nitrogen.
3. Incubation is made in a 37°C water bath under a normal atmosphere.
4. An algal protein hydrolysate labeled with C^{14} is used as a source of amino acids.

The ribosomes isolated in the above manner are very stable in liquid nitrogen and decay slightly at −20°C. They incorporate five to eight times better than ribosomes prepared by previous methods, and it appears that the use of bentonite and the centrifugation of the aggregated material at 30,000 × *g* removes the ribonuclease, active and latent, that has destroyed the activity of these particles in the past.[4] The aggregated material comprises about 50% of the ribosomes initially collected at 105,000 × *g* and contains 16% RNA. It is relatively inactive in the incorporation of C^{14} amino acids but does contain most of the particle-bound antibody when isolated from immunized animals. The ribosomes of the 105,000 × *g* centrifugation for 60 min contain 45 to 50% RNA and do not have hemagglutinin activity until 24 hr after injection.

Ribosomes have been prepared from male rats that were immunized to sheep erythrocytes at 4, 8, 12, 16, 20, and 24 hr after injection as well as from nonimmunized animals. The relative rate and maximal amount of incorporation of C^{14}-labeled amino acids per mg of ribosomes increase significantly after the injection of antigen even at 4 hr. A nonimmunized liver supernatant protein is used as a standard source of sRNA and enzymes.

Ribosomes isolated 4 to 24 hr after the injection of antigen show hemagglutinin activity after incubation in the incorporation mixture. This increase in visually assayed hemagglutination is correlated with an increase in the amount of acid-precipitable C^{14} bound to sheep erythrocytes after thorough washing in Veronal buffer supplemented with 2% normal rat serum.

Ribosomes taken at 16 and 24 hr have been examined in the Spinco model E ultracentrifuge and found to have a range of sizes – 81s, 107s, 120s (the predominant group), 151s, and 174s. After incubation, the mixture of ribosomes and components necessary for incorporation was centrifuged in a sucrose density gradient from 10 to 34%. Fractions were collected, and the $O.D._{260}$, acid-precipitable radioactivity, and the ability to hemagglutinate sheep erythrocytes were determined. Most of the radioactivity was associated with the 120s fraction and the hemagglutinating activity with the 151s ribosomes. When sheep erythrocytes were added to the incubation mixture prior to gradient centrifugation, the hemagglutinating ability was lost from the

151s ribosomes and associated with the erythrocytes positioned at the interface between the gradient and a small added amount of 60% sucrose at the bottom of the tube. There was a corresponding loss of radioactivity from the 151s ribosomes that was found associated with the erythrocytes at the interface.

These results indicate that there is ribonucleoprotein-bound antibody as early as 4 hr after the injection of antigen and that ribosomes isolated at 4 hr and thereafter can synthesize antibody in a cell-free system.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]C. J. Wust, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 138.

[3]C. J. Wust, this report, chap. 17.13.

[4]C. J. Wust and G. D. Novelli, *Arch. Biochem. Biophys.* 104, 185–191 (1964).

17.15 ISOLATION AND PURIFICATION OF SPECIFIC TRANSFER RNA

A. D. Kelmers[1] G. D. Novelli
M. P. Stulberg

Introduction. – In order to study the biochemical parameters and the physical and chemical properties of transfer RNA (sRNA), large amounts of the individual amino acid sRNA's must be prepared. We have initiated a collaborative effort with the Chemical Technology Division to attain this goal. Their knowledge and background in the isolation of significant quantities of various chemical entities by large-scale procedures promises to be of considerable aid in this project. Chromatography, solvent extraction, and other methods will be employed to attain an ultimate separation and purification of the various sRNA molecules.

Results and Discussion. – The initial goal is to obtain large quantities of sRNA free of other nucleic acids, proteins, etc. A large number of different methods presently employed to purify sRNA have been tested and analyzed. It appears that a combination of phenol extraction, isopropanol precipitation, and DEAE chromatography will produce high yields of active preparations of sRNA. These methods will meet the requirements of a pilot-plant operation that will produce stock quantities of sRNA sufficient for further use in the separation and purification of all existing sRNA molecules. This project will also serve as a valuable aid in the preceding project as well as others now existing in the Biology Division.

[1]Chemical Technology Division.

17.16 ISOLATION AND PURIFICATION OF AMINO-ACID-ACTIVATING ENZYMES

M. P. Stulberg

Introduction. – Transfer RNA (sRNA) plays an important role in the metabolism of the cell. It is an obligate intermediate in the pathway of protein synthesis and is responsible for much of the specificity and control displayed in the biosynthesis of polypeptides. In order to elucidate the mechanism of sRNA interaction with the amino-acid-activating enzymes, these enzymes must be purified to a high degree. Once this goal is accomplished, the following questions can be examined. How do the activating enzymes recognize the correct sRNA for amino acid transfer? Is the nucleotide code that is responsible for specific binding of sRNA to messenger RNA on the response also operative in sRNA-enzyme interaction? Can homogeneous species of sRNA be isolated with the utilization of purified activating enzymes as an isolation device? What are the properties of homogeneous preparations of purified activating enzymes? Does the cell utilize the sRNA as a control and regulating device in nucleic acid function and protein biosynthesis? Is variation in control of sRNA function a significant factor in cancer induction?

Results and Discussion. – RNA polymerase is currently being prepared in large quantities by the use of the pilot-plant fermenter for production of large quantities of *E. coli* as starting material. A by-product of the polymerase preparation contains large amounts of the amino-acid-activating enzymes. We have chosen this source as our

starting material for enzyme isolation. A valid rate assay has been developed that is dependent on amino acyl–sRNA formation. Enzyme-containing preparations have been fractionated by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography prior to the use of other methods to purify the leucine- and isoleucine-activating enzymes.

17.17 PILOT-PLANT OPERATION

E. F. Phares J. T. Davidson
Mary V. Long

Pilot-plant operations during this period have been directed toward refinement of technique with the 100-gal fermenter. We had to gain experience with respect to size of inoculum and time of day to inoculate so that harvesting of the fermenter could be carried out largely during normal working hours. Since a variety of organisms are grown in the fermenter, these parameters had to be determined for each organism. We also had to devise methods for harvesting the cells from the fermenter. The rate at which a culture can be passed through the Sharples centrifuge varies with the size of the organism; so these conditions had to be determined for each organism grown. The organisms grown, together with the yield and the number of runs, are listed in Table 17.17.1.

In addition, six runs were not harvested because of contamination. Two of these were *E. coli* B in which the stock cultures were found to be contaminated with a bacteriophage. One batch was contaminated by an accident during inoculation. An aerobic sterility test with an enriched medium was carried out for three days with satisfactory results.

We expect to produce rather large quantities of *E. coli* B that will serve as a source of sRNA in the joint program with Chemical Technology.

HORMONAL CONTROL

Several approaches are being used in our studies on the mechanism of control of protein synthesis by steroid hormones. The in vivo changes in RNA synthesis that occur rapidly upon steroid administration are being studied in two hormone: target systems, namely, hydrocortisone in the liver and testosterone in the seminal vesicles. In vitro studies of possible steroid–nucleic acid interactions have been attempted employing both physical and enzymatic analyses. A system of intermediate complexity in which steroid effects can be observed is now available in the Reuber hepatoma cell line, which is being grown in tissue culture in preparation for studies of hormonal control in this tumor. This presents an

Table 17.17.1. Summary of Fermenter Operations

Organism	Number of Runs	Total Yield (kg)	Group
Azotobacter vinlandii	3	8.0	Enzymology
Clostridium butyricum	1	1.2	Enzymology
Clostridium pasteurianum	9	16.4	Enzymology
Desulfovibrio desulfuricans	8	2.3	Enzymology
Escherichia coli B	2	4.6	Enzymology
E. coli lac^-	3	10.1	Enzymology
E. coli leu^-, tyr^-, ser^+	1	0.1 (small bio.)	Nucleic acid enzymology
Bacillus megaterium (spores)	1	0.1	Biophysics
Streptococcus faecalis	1	1.2	Microbiology

opportunity to compare the mechanisms of regulation in normal tissue with those in a neoplastic "mutant" of the same tissue.

17.18 STIMULATION OF NUCLEAR RNA SYNTHESIS BY HYDROCORTISONE[1]

D. L. Greenman F. T. Kenney

Introduction. – We have previously described[2] how administration of hydrocortisone to adrenalectomized rats effects a three- to fivefold stimulation of the rate of nuclear RNA synthesis. Analyses of size distribution, messenger activity, and base composition indicated that the hormone caused little or no change in these parameters. These results suggested that the hormone effect was not limited to one type of RNA but was more general in nature. In the present work we have further analyzed the functional roles of the RNA's synthesized in pulse-labeling experiments, using concepts and techniques described by Perry[3] and by Georgiev *et al.*[4]

Results and Discussion. – Liver nuclear fractions from adrenalectomized rats given P^{32} (1 mc) for 20 min before death were extracted by a differential phenol extraction[4] that yields four RNA fractions. These have been characterized as follows: *Fraction 1* contains most of the unlabeled RNA and only about 6% of the labeled RNA. The isotope is all found in the 4S region on density-gradient centrifugation, and thus we consider this fraction to be largely contaminating cytoplasmic RNA. *Fraction 2* (obtained at 45°) contains about 25% of the newly synthesized RNA, which in base composition resembles ribosomal RNA $[(A + U)/(C + G) = 0.72]$; this RNA is synthesized in nuclear regions[3] and is a precursor to ribosomal RNA.[3,4] *Fraction 3* (obtained at 64°) contains 40% of the labeled RNA, and in base composition $[(A + U)/(C + G) = 0.92]$ appears to contain roughly equal amounts of ribosomal precursor RNA and a DNA-like RNA. *Fraction 4* is obtained by extracting at 80° in the presence of detergent and contains about 30% of the total radioactivity. Base composition is very similar to DNA. On sucrose gradients the labeled material appears to be larger than any of the other RNA species, labeled or unlabeled, and it therefore appears that this may be intact messenger RNA. Confirmation of this awaits in vitro experiments now in preparation.

From these results and those of others[3,4] it is apparent that the pulse-labeled RNA of liver nuclei contains two types of RNA: (1) a ribosomal precursor, synthesized in nucleoli, high in guanine and cytosine residues, and extractable at moderate temperatures; and (2) a DNA-like RNA, high in adenine and uracil content, and extracted at high temperatures with the aid of detergent. This fits our original report[2] on the base composition of the total pulse-labeled nuclear RNA. Since we found that this composition was unchanged by hormone treatment, it is apparent that the hormone must accelerate the synthesis of both of these components. Experiments are in progress to confirm this assumption. Should confirmation be forthcoming, these experiments will demonstrate that the simple gene activation model of enzyme induction is not sufficient to explain the mechanism of hormonally mediated inductions.

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[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]F. T. Kenney and F. J. Kull, *Proc. Natl. Acad. Sci. U.S.* **50**, 493 (1963).

[3]R. Perry, *Journal of the National Cancer Institute*, in press.

[4]G. P. Georgiev *et al.*, *Nature* **200**, 1291 (1963).

17.19 STEROID–NUCLEIC ACID INTERACTIONS[1]

D. L. Greenman F. T. Kenney

Introduction. – The growth response of target organs to specific steroid hormones is generally characterized by increased protein synthesis preceded by an increase in the rate of RNA synthesis. Inhibition of this steroid-stimulated RNA synthesis by administration of actinomycin indicates that the RNA synthesized in response to hormone treatment is DNA directed and further suggests the possibility of direct DNA-steroid interaction as a basic mechanism of hormone action.

Results and Discussion. – Preliminary studies have been initiated for the purpose of detecting possible in vitro DNA interactions with steroid hormones. In the present study two observations have suggested that such interactions may exist: (1) Ultraviolet absorption (240–300 mμ) of a mixture of estradiol 17-B and DNA or DNA-nucleoprotein (prepared from calf thymus) is less than the additive absorption of the nucleic acid and steroid when determined separately. (2) The interaction of actinomycin with DNA appears to be altered in the presence of estradiol. This is indicated by the observation that the absorption of actinomycin at 425 mμ is lowered less by addition of a mixture of DNA and estradiol than by the addition of DNA alone.

The effects of steroids on the melting temperature and DNase digestion of DNA-P have also been studied. Results from these studies have been variable and generally indicate little or no effect. Similarly, the activity of DNA-P in stimulating RNA synthesis by an active DNA-dependent RNA polymerase preparation has been unaltered by addition of hydrocortisone to the reaction mixture.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

17.20 EFFECTS OF TESTOSTERONE ON RNA SYNTHESIS IN RAT SEMINAL VESICLE[1]

W. D. Wicks F. T. Kenney

Introduction. – It has been shown that the synthesis of RNA in the seminal vesicle of castrated rats is markedly increased following a single injection of testosterone.[2] The present studies were designed to determine the earliest time when the hormone affects the synthesis of RNA and to assess the nature of the newly synthesized material.

Results and Discussion. – Adult rats castrated 12 to 15 hr previously were treated with testosterone for different periods of time and injected with 3 mc of P^{32} 50 min before they were killed. The seminal vesicles were homogenized, and RNA was extracted from the acid-insoluble residue with hot salts. Contaminating material was removed by the use of DEAE-paper and charcoal columns. The specific activity of the isolated RNA was corrected for variations in the extent of labeling of precursor pools. Within 20 min after administration of the hormone, a 50% increase in the specific activity of RNA could be detected. The specific activity rose sharply until approximately 60 min, when a two- to threefold increase was obtained. No further increase could be detected as long as 240 min after the hormone was given. Base composition analyses of the pulse-labeled RNA revealed that (1) the composition was intermediate between ribosomal and DNA-like RNA $[(A + U)/(G + C) = 1.06]$; (2) the labeled RNA composition was radically different from that of the bulk RNA determined spectrophotometrically $[(A + U)/(G + C) = 0.63]$; and (3) there was no alteration in the composition at any interval after the administration of the hormone. Efforts to localize the cellular site of hormone action have been hindered by difficulties encountered in the centrifugal fractionation of this tissue. When attempted, however, this procedure revealed that a hormone-induced increase occurred in both the nuclear and cytoplasmic fractions, but it is not known in which fraction the increase was initiated. In present experiments we are attempting to fractionate the labeled RNA by other means in hopes of determining whether an increase in the synthesis of all types of RNA is induced by testosterone or if only certain species are involved.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]W. D. Wicks and C. A. Villee, "Studies on the Course of Action of Testosterone Propionate on the Rat Seminal Vesicle," submitted to *Archives of Biochemistry and Biophysics*.

17.21 HORMONAL CONTROL MECHANISMS IN A RAT HEPATOMA[1]

F. T. Kenney R. L. Tyndall[2]

Through the courtesy of Drs. Paul Morse and Van Potter of the University of Wisconsin, we

have obtained samples of the Reuber H-35 hepatoma, a tumor derived from rats and the first "minimal deviation" hepatoma to be grown in tissue culture. These cells have been shown to respond to adrenal steroid hormones by a rapid induction of the tyrosine transaminase. We are now attempting to repeat this finding, with a view toward characterizing the response more fully than has been done previously. When these preliminary experiments are completed, studies on the changes in RNA metabolism that precede enzyme induction (*vide supra*) will be extended to these cultures. Many experiments that we have been unable to do previously, using rats, will be possible with the cultured cells. Since our experiments deal directly with aspects of the control of growth processes, extension to a system derived from the same tissue but lacking full control may lead to a better understanding of abnormal as well as normal growth.

[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]Pathology and Physiology Group.

MAMMALIAN CELL REGULATION

The reports on mammalian cell regulation constitute a continuing study of the lactic dehydrogenase isozymes. We are attempting to understand the reasons 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.

17.22 LACTIC DEHYDROGENASE PURIFICATION

T. H. Turney, Jr. K. B. Jacobson

Introduction. – The resolution of lactic dehydrogenase (LDH) into multiple subbands by electrophoresis on polyacrylamide gels has been described.[1] A theory involving differential coenzyme binding on the monomeric units of the LDH molecule was advanced as an explanation for the appearance of multiple subbands. Another interpretation involving genetic factors has been put forth by Costello and Kaplan[2] explaining the appearance of multiple subbands.

Both interpretations are not without supporting data; however, the work to date has been limited to isolated observations, and the data themselves are not clear in some cases. It seemed reasonable that both a qualitative and a quantitative examination of the amino acid content of the individual subbands would indicate whether or not their appearance was due to genetic factors.

Results and Discussion. – Different pig, beef, and rabbit tissues were electrophoretically examined to determine which tissue presented major bands of activity that could be divided into subbands of equal or nearly equal intensity. The electrophoretic pattern of rabbit liver LDH appears to satisfy this condition.

A method of LDH purification is being established which involves the separation of the major LDH isozymes by salt-gradient elution from a DEAE column at pH 7.0 and their subsequent purification by ammonium sulfate precipitation.

[1]P. J. Fritz and K. B. Jacobson, *Science* **140**, 64 (1963).

[2]L. A. Costello and N. O. Kaplan, *Biochim. Biophys. Acta* **73**, 658 (1963).

17.23 OBSERVATIONS ON MULTIPLE MOLECULAR FORMS OF LACTIC DEHYDROGENASE[1]

K. B. Jacobson T. H. Turney, Jr.
P. J. Fritz J. B. Murphy

Introduction. – The resolution of the individual isozymes of lactic dehydrogenase (LDH) into multiple fractions by electrophoresis in polyacrylamide gels has been described in a previous report.[2] A theory involving differential coenzyme binding was proposed to account for this phenomenon.[3]

Results and Discussion. – The first observation of multiple banding in a single isozyme was made in the organs of the mouse. Rabbit organs

also contain LDH isozymes that break up into multiple bands in electrophoresis, and it was established that crystalline LDH from rabbit muscle behaved essentially in an identical fashion as the crude homogenate. The LDH isozymes of beef, pig, and chicken do not exhibit the multiple bands characteristic of the mice and rabbits; so this phenomenon appears to be restricted to certain species. In contrast the occurrence of the five major isozymes is widespread in the vertebrate and invertebrate animals. Evidence from various laboratories supports a hypothesis to explain the occurrence of the major isozymes; the LDH molecule consists of four peptide subunits of which there are two structurally distinct types. Combinations of these two types in groups of four yield five separate products if sequential order is not considered. The discovery that each of these five isozyme forms is further subdivided presents a challenge to the theory as presently proposed.

Conditions for reproducible observation of the multiple bands of isozymes have been explored. Fairly high voltage gradients (26 v/cm) on the polyacrylamide gel are necessary, presumably to minimize the time for diffusion during separation. At pH 8.5 both mouse and rabbit LDH isozymes are best resolved into subbands. The mouse subbands are sharpest when 2-mercaptoethanol (0.003 *M*) is present in the gel during electrophoresis, but resolution of the rabbit subbands is not affected. At higher mercaptoethanol concentrations (0.007 *M*) the subband structure in both mouse and rabbit appears to condense and become obscure. The pH optimum and mercaptoethanol effect may be used to suggest that the ionization of –SH groups has a pronounced effect on the resolution of subbands.

Another difference between the mouse and rabbit became apparent when nicotinamide adenine dinucleotide (NAD) was included in the gel. This experiment was to test the hypothesis that partially complete binding of this coenzyme was the reason for the subbanding of the isozymes. Including 0.01 *M* NAD in the gel had no effect on the subbands of the rabbit but appeared to condense the subbands of the mouse. The latter fact supported the coenzyme binding explanation of subband occurrence in the mouse but was not applicable to the rabbit. An alternative hypothesis by Costello and Kaplan suggests a genetic difference in the subbands of the mouse.

Neither the coenzyme binding nor the genetic hypothesis is consistent with more recent facts. The number of subbands found in mouse organs is too great to be consistent with either explanation, and in the case of the rabbit the distribution of subbands is not correct. Furthermore, individual subbands of the mouse are either converted from one to another or have reciprocal activation-inhibition responses to mercaptoethanol; the former possiblity would be inconsistent with the genetic explanation. Further efforts to elucidate the story of the subfractionation of LDH isozymes are being made.

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[1]Cooperative NIH-AEC Chemical Co-Carcinogenesis Program.

[2]P. J. Fritz and K. B. Jacobson, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp. 142–43.

[3]P. J. Fritz and K. B. Jacobson, *Science* **140**, 64 (1963).

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.

17.24 SULFATE REDUCTION AND PHOSPHORYLATION IN CELL-FREE PREPARATIONS FROM *DESULFOVIBRIO DESULFURICANS*

Sister M. Regina Lanigan H. D. Peck, Jr.

Introduction. – It has been postulated that *Desulfovibrio desulfuricans* obtain the energy required for growth during the reduction of SO_4^{2-} with hydrogen by an "anaerobic" oxidative phosphorylation; however, the evidence for this oxidative phosphorylation is largely indirect, being derived mainly from growth[1] and inhibitor[2] studies. Direct evidence for this reaction, the demonstration in extracts of phosphorylation coupled to electron transport, has not as yet been obtained, although a number of assay systems have been examined.

Results and Discussion. – In the course of investigating the effect of fluorophosphate on

SO_4^{2-} reduction with hydrogen and ATP in extracts, a small endogenous reduction of SO_4^{2-} was observed to be inhibited by fluorophosphate. As the oxidative phosphorylation and not the ATP dependent reduction of sulfate is presumably inhibited by fluorophosphate,[3] the nature of the endogenous reduction of sulfate is being examined.

This reduction of sulfate occurs only in extracts prepared from cells that have been well washed and freed of FeS. In addition, greatest activities are obtained in extracts prepared from young cells (1–2 days) and broken in the French pressure cell at comparatively low pressure (7000 psi). After treating freshly prepared extracts with DNase, it is possible to remove most whole cells and obtain two particle fractions by differential centrifugation. The lighter of the two particle fractions exhibited most of the sulfate-reducing activity sensitive to fluorophosphate, although it contained few, if any, whole cells. These particles completely lost activity in 24 hr and, after washing, required the addition of supernatant protein for activity.

After better defining conditions for this ATP-independent reduction of sulfate, the preparation will be examined directly to determine whether oxidative phosphorylation is actually occurring and thereby supplying ATP for the reduction of sulfate.

[1]J. C. Senez, *Bacteriol. Rev.* **26**, 95 (1962).

[2]H. D. Peck, Jr., *J. Biol. Chem.* **235**, 2734 (1960).

[3]Sister M. Regina Lanigan and H. D. Peck, Jr., *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 189.

17.25 STUDIES ON THE MECHANISM OF ACTION OF ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE

H. D. Peck, Jr. J. T. Davidson

Introduction. – Adenosine 5′-phosphosulfate reductase (APS) from *Desulfovibrio desulfuricans* is an iron-containing flavoprotein (mol. wt 150,000) that catalyzes the mononucleotide-dependent oxidation of SO_3^{2-} to APS as follows: $AMP + SO_3^{2-} \rightleftharpoons APS + 2e$. Several observations have suggested that this oxidation is a two-step reaction involving an "enzyme-sulfur" as an intermediate.[1] Although it was possible to demonstrate the formation of S^{35}-labeled protein after exposure of the enzyme to sulfite in the absence of AMP, it has not until now been possible to demonstrate that this labeling of APS reductase was related to enzymatic activity.

Results and Discussion. – The paper disk method,[2] utilized in the study of amino acid incorporation into protein, has been slightly modified and employed to determine the incorporation of $S^{35}O_3^{2-}$ into APS reductase. By use of this technique it was possible to demonstrate that the kinetics of S^{35} labeling are similar to those of the reduction of enzyme-bound FAD by SO_3^{2-} in the absence of added AMP. If the S^{35}-labeled protein is to be considered as an intermediate, this label should be removed or decreased in amount when the labeled enzyme is incubated with AMP. By use of the disk assay, it was shown that half of the S^{35} label was removed from the enzyme after incubation with AMP, GMP, or IMP. These three nucleotides also function as sulfate acceptors in the activity assay; however, nucleotides that do not function as sulfate acceptors in the activity assay show little or no activity in removing S^{35} from APS reductase.

These results indicate that at least a portion of the enzyme-bound S^{35} is related to enzymatic activity and, in addition, may indicate the formation of two "enzyme-sulfur" intermediates in this oxidation.

[1]H. D. Peck, Jr., and J. T. Davidson, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, pp. 143–44.

[2]R. J. Mans and G. D. Novelli, *Arch. Biochem. Biophys.* **94**, 48 (1961).

17.26 SEQUENTIAL COUPLING OF PHOSPHORYLATION AND CARBON DIOXIDE FIXATION IN EXTRACTS OF *THIOBACILLUS THIOPARUS*

E. J. Johnson H. D. Peck, Jr.

Introduction. – Both the formation of ADP from APS produced during the oxidation of SO_3^{2-} to SO_4^{2-} (ref 1) and the ATP-dependent fixation of $C^{14}O_2$ (ref 2) have been demonstrated to be present in extracts of *Thiobacillus thioparus*. As the relationship between phosphorylation and

CO_2 fixation in these organisms has been a subject of controversy,[3] it was proposed to demonstrate quantitatively and sequentially the formation of high-energy phosphate during the oxidation of sulfite and its subsequent utilization for CO_2 fixation.

Results and Discussion. – Extracts of *T. thioparus* were prepared by means of a French pressure cell, and after removal of small molecules by gel filtration, $C^{14}O_2$ fixation was completely dependent upon the presence of ATP, Mg^{2+}, and ribose 5-phosphate. The amount of $C^{14}O_2$ fixed, in terms of specific activity, was at least tenfold greater than that previously reported for those microorganisms. In addition to ATP, ADP and a variety of nucleotide triphosphates (ITP, GTP, UTP, and dATP) served with varying degrees of effectiveness for the fixation of CO_2.

As the CO_2-fixing system in autotrophic organisms utilizes a major portion of the energy produced by the cell, it is of interest that AMP very strongly inhibits CO_2 fixation. This may conceivably represent a control mechanism for preventing the complete utilization of high-energy phosphate by the CO_2-fixing system.

In spite of this inhibition of CO_2 fixation by AMP, it was possible to partially replace the ATP required for CO_2 fixation with SO_3^{2-}, PO_4^{3-}, AMP, and air. Phosphorylation and $C^{14}O_2$ fixation were separated in time by first incubating SO_3^{2-} and AMP aerobically and then, after flushing with helium, introducing $C^{14}O_3^{2-}$ and ribose 5-phosphate into the reaction mixture. During the first incubation $P^{32}O_4^{3-}$ was utilized for the synthesis of nucleotides, mainly ADP; and in the second incubation, $C^{14}O_2$ was fixed and the esterified phosphate was utilized in an amount equal to the CO_2 fixed. The data obtained with this system provide the first in vitro evidence for the mechanism of coupling CO_2 fixation and phosphorylation in *T. thioparus*.

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[2]E. J. Johnson and H. D. Peck, Jr., *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 188.

[3]S. R. Elsdey, p. 5 in *The Bacteria*, vol. III, Academic Press, New York, 1962.

18. Microbiology

Enzyme and Tracer Studies on Bacterial Metabolism

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18.1 A MONOSUBSTITUTED NAPHTHOQUINONE FROM *STREPTOCOCCUS FAECALIS*, 10CI: FURTHER CHARACTERIZATION AND LARGE-SCALE PREPARATION

R. H. Baum M. I. Dolin

After preliminary data[1–3] established the presence of a quinone (SFQ) in the lactic acid bacterium *Streptococcus faecalis*, strain 10Cl, further characterization[4] led to its identification as a monosubstituted 1,4-naphthoquinone, containing a β-alkenyl side chain, 40 to 45 carbon atoms long.

Because of the small amount (1 to 2 $\times$ 10^{-4} micromole per mg of dry weight of cells) of SFQ present in the organism, characterization has been carried out by microanalytical procedures. Complete structural identification of the side chain will require larger amounts of pure SFQ. Thus, large-scale preparation of the quinone is currently being carried out through the use of the Pilot Plant Facility[5] available in the Division. This will provide sufficient compound for characterization of the quinone by nuclear magnetic resonance, infrared spectroscopy, hydrogenation, quantitative chemical analysis, etc. Large-scale chemical synthesis of known β-alkenyl-1,4-naphthoquinones are also under way. These compounds are necessary as standards for some of the physicochemical determinations.

It has been found that 2-phytyl- and 2-farnesyl-1,4-naphthoquinone, chemically synthesized by the method of Fieser *et al.*,[6] modified by Hirschmann *et al.*,[7] have ultraviolet spectra virtually identical to that of SFQ. Further, these chemically synthesized quinones react with dilute alkali in ethanol to give the violet chromophore characteristic of monosubstituted β-alkenyl-1,4-naphthoquinones. The absorption maxima and molecular extinction coefficients of these alkali-produced chromophores are the same as those given by the alkali product of SFQ. These data confirm the presence of a β-alkenyl linkage in the latter.

Preparation of SFQ on a large scale will allow characterization of the side chain beyond the β-alkenyl linkage and provide sufficient compound for biochemical and physiological experiments designed to determine the metabolic role of this new quinone in the microorganism.

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[2]R. H. Baum and M. I. Dolin, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1963*, ORNL-3427, p. 146.

[3]R. H. Baum and M. I. Dolin, *Biol. Div. Semiann. Prog. Rept. Aug. 15, 1963*, ORNL-3498, p. 191.

[4]R. H. Baum and M. I. Dolin, *J. Biol. Chem.* **238**, PC4109 (1963).

[5]E. Phares *et al.*, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 175.

[6]L. F. Fieser, M. Tishler, and N. L. Wendler, *J. Am. Chem. Soc.* **62**, 2861 (1940).

[7]R. Hirschmann, R. Miller, and N. L. Wendler, *J. Am. Chem. Soc.* **76**, 4592 (1954).

18.2 OPTICAL ACTIVITY ATTRIBUTABLE TO CONFORMATION IN A PYRIDINE DINUCLEOTIDE

M. I. Dolin K. B. Jacobson[1]

The conformations assumed by pyridine nucleotides in solution may strongly influence their catalytic activity. For instance, the folded conformation of NADH[2] makes the hydrogen atoms in the 4 position stereochemically nonequivalent. Changes in pyridine nucleotide conformation that accompany reduction and reoxidation of the molecule help to explain certain features of hydrogen transport at the pyridine nucleotide level.[3] As an example of the manner in which chemical structure affects the conformation of pyridine nucleotides, we have been studying the oxidized form of NAD-acetone.[4,5] The spectral and fluorimetric properties of this molecule indicate that it exists in a folded conformation because of complex formation between the adenine moiety and the modified pyridine ring. Confirmation of the existence of such a complex has come from nuclear magnetic resonance studies carried out in collaboration with O. Jardetzky. The data show that there is very strong anisotropic shielding of the adenine and pyridine ring protons, indicating the existence of a stable J_1-J_1 complex.

Folded conformations have been described for other dinucleotides;[2] however, one consequence of intramolecular complex formation in oxidized NAD-acetone has not been noted with other compounds. The folded conformation of the oxidized acetone adduct causes the optical rotation to change by 40,000°/mole, as illustrated by the following molecular rotations (pH 7–7.3): oxidized NAD-acetone (+26,600); oxidized NMN-acetone (+5340); and AMP, derived from diesterase cleavage of oxidized NAD-acetone (–18,800). These data show that the positive rotation of oxidized NAD-acetone cannot be accounted for by the sum of the rotations of the constituent moieties (–13,460) but must be attributable to some feature of the dinucleotide conformation.

It is possible that the positive rotation is caused by the folded conformation itself. A model is afforded by hexahelicene.[6] Because of molecular overcrowding, this molecule exists in either of two helical conformations. Both isomers have been isolated and are strongly optically active. It may be noted also that the positive rotation of undenatured nucleic acid (+40,000° per phosphate residue) is attributed to the helical conformation of the macromolecule.[7] An alternative hypothesis is that the conformation of the ribose attached to the pyridine ring of the acetone adducts affects the optical rotation. Studies in progress should allow a choice between the two hypotheses.

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18.3 BOUND PYRIDINE NUCLEOTIDE OF MALIC-LACTIC TRANSHYDROGENASE

M. I. Dolin E. F. Phares
Mary V. Long[1]

Several unique properties of malic-lactic transhydrogenase make this enzyme useful for the study of hydrogen transport at the pyridine nucleotide level. The reaction catalyzed by the enzyme has been described in the previous report.[2]

The purified enzyme (mol. wt $\sim 1 \times 10^5$) is highly fluorescent and contains firmly bound NAD and NADH in a ratio of approximately 0.3/1. The bound NADH is 15 times as fluorescent as free NADH. Approximately 3 moles of total pyridine nucleotide are present per 1×10^5 g of protein. The enzyme has a broad absorption band between 310 and 400 mμ. On the addition of pyruvate, the band is partially bleached, and the difference spectrum shows a peak at 370 mμ, this change being accompanied by complete quenching of fluorescence and by intensification of the absorbance in the region of 320 mμ. After removal of pyruvate by dialysis, the fluorescence returns to 83% of its initial value; however, analysis of the oxidized enzyme shows that approximately 40%

of the bound NADH has been oxidized to bound NAD. The bound NADH of the oxidized enzyme is 22 times as fluorescent as free NADH. The fluorescence of the pyruvate-free oxidized enzyme is completely quenched on the further addition of pyruvate. On the addition of malate to the oxidized pyruvate-free enzyme, the spectrum of the original unoxidized enzyme is restored, and the fluorescence returns to 95% of its original value.

Preliminary experiments performed in collaboration with Gregorio Weber demonstrate that a portion, at least, of the tryptophan in the enzyme is located within 25 A of the NADH binding sites. Energy transfer takes place from the excited state of tryptophan to DPNH, by a Förster[3] process.

The data summarized above imply that the bound NADH molecules are not equivalent. Present information suggests that there are at least two species of bound NADH; these differ in their oxidizability by substrate and in the quantum yield of their fluorescence. The emission spectra of the bound NADH and of the tryptophan suggest that the NADH binding sites are strongly shielded and surrounded by a medium that is less polar than water. This situation fits well with the known high substrate specificity of the enzyme and with the finding that enzyme-bound NADH is not accessible to compounds which oxidize NADH nonenzymically.

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[1]Miss Long's portion of this work was performed before she transferred to the Enzymology Group.

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19. Plant Physiology and Photosynthesis

Plant Physiology (19.1–19.4)		Photosynthesis (19.5)	
A. H. Haber	Pat C. Kerr	W. A. Arnold	J. R. Azzi
D. E. Foard	N. Ann Payne	W. F. Bertsch	
T. J. Long[a]	Helen J. Luippold[b]	M. A. Kamrin[c]	

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19.1 IS FINAL SIZE DETERMINED BY RATE AND DURATION OF GROWTH?

A. H. Haber T. J. Long
N. Ann Payne

Introduction. – Little is known of the biological relations among the extent of growth, duration of growth, and growth rate[1] for determinate growth in plants. These quantities are, of course, related by the mathematical equation

$$R = \frac{s_2 - s_1}{t_2 - t_1},$$

where s_1 is the size of a structure already present at zero time, s_2 the size of the structure after it has stopped growing, t_1 the time after zero time when growth begins, and t_2 the time when the structure reaches its final size of s_2 and its growth stops. The quantity R is thus defined as the average growth rate within the time interval between t_1 and t_2, which interval is the duration of growth.

Different final extents of growth $(s_2 - s_1)$ have been interpreted as being due to changes in either or both growth rate R and duration[1–3] $(t_2 - t_1)$. Of course $(s_2 - s_1)$ is in a *mathematical* sense determined by R and $(t_2 - t_1)$. One might with equal justification, however, state that $(t_2 - t_1)$ is mathematically determined by $(s_2 - s_1)$ and R and that R is mathematically determined by $(s_2 - s_1)$ and $(t_2 - t_1)$. The interpretation that $(s_2 - s_1)$ is in a *biological* sense determined by R and $(t_2 - t_1)$ holds the implicit assumption that $(s_2 - s_1)$ is the dependent variable whose values are fixed by the independent variables R and $(t_2 - t_1)$. The implicit assumption is that $(s_2 - s_1)$ is merely secondarily determined biologically as a consequence of the relatively more primarily determined values of R and $(t_2 - t_1)$.

This paper presents evidence unfavorable for the interpretation that final size in determinate growth should generally be attributed to rate and duration of growth. The system studied is the growth of the epiblast, a small structure which, with the coleorhiza, forms a morphological structure peculiar to grasses.[4,5] The wheat epiblast is ideally suited for certain morphogenetic studies emphasizing the determinate aspects of plant growth for the following reasons:

1. During germination the epiblast can be considered a most extraordinarily determinate structure. All the cells finally present at cessation of its growth are already present in the embryo of the mature grain.[5]

2. The epiblast-coleorhiza is the first structure to grow during germination of grasses.[5,6] Consequently, in studies of factors affecting epiblast growth during germination, one can neglect the usual complication that its growth may be partly

affected by influences on prior growth in other parts of the embryo or seedling during the experiment.

3. The wheat epiblast is an appendicular structure that, except for its contiguity with the coleorhiza, is isolated from the rest of the plant. Thus, its behavior is little constrained by contact with other parts of the embryo or seedling. In contrast to other experimental systems of comparable simplicity in this regard, there is no surgical excision with its attendant complications.

4. The epiblast is histologically simple, consisting of only cortical parenchyma surrounded by an epidermis.[6,7]

Results. – Growth, in terms of epiblast breadth, was studied during germination of wheat at various temperatures. Both the average growth rate R and the duration of growth $(t_2 - t_1)$ greatly depend on temperature between 5.5 and 23.5°C. Although both R and $(t_2 - t_1)$ vary approximately fifteenfold, s_2 is unaffected by these variations in temperature. In view of this invariance of s_2, and therefore of $(s_2 - s_1)$, despite the great differences in R and the great differences in $(t_2 - t_1)$, there is no justification for assuming that $(s_2 - s_1)$ is the dependent variable whose value is fixed by the independent variables R and $(t_2 - t_1)$. It appears that in this instance it is $(s_2 - s_1)$ itself, rather than R and $(t_2 - t_1)$, that is primarily determined biologically. Thus it seems most unlikely that epiblast size at maturity is merely the secondary result of more-primary influences upon the rate and upon the duration of epiblast growth during germination.

Discussion. – Because of these results, one might question the interpretation that the extent of determinate growth generally is due to the rate and duration of growth. We do not imply that in determinate growth final size is always unaffected by temperature or other environmental factors. It remains questionable, however, whether such changes in the extent of growth, which are expressed as differences in rate and/or duration of growth, are caused by means of the differences in rate and/or duration of growth. Neither do we imply that in determinate growth there are no instances in which agents that affect rates and/or duration of growth do not thereby causally influence the extent of growth. In general, however, it is certainly unjustified and very probably incorrect to assume that in determinate growth the ultimate increase in size is a biological consequence of the rate and duration of growth.

In conclusion, our answer to the question which forms the title of this report depends upon whether the word "determined" is used in the mathematical or the biological sense. Growth is always mathematically determined by its rate and duration. This last statement is biologically trivial, however, since any two of the quantities $(s_2 - s_1)$, R, and $(t_2 - t_1)$ mathematically determine the third. By contrast, however, it is not true that the ultimate extent of growth of determinate structures is always biologically determined by the rate and duration of growth. Thus, although for a given set of environmental conditions both the rate and the duration of growth may be considered to be ultimately under genetic control, such genetic controls of rate and duration of growth are not, in general, the means by which growth itself is determined biologically.

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19.2 RADIORESISTANCE OF GROWTH IN CERTAIN SYSTEMS WHICH GROW WITHOUT CELL DIVISION IRRESPECTIVE OF IRRADIATION

A. H. Haber D. E. Foard
Helen J. Luippold

Introduction. – The growth (expansion) of an organ is the summation of the expansions undergone by its constituent cells; this identity is true irrespective of the presence, absence, or extent of cell division. Growth and cell division are, nevertheless, interrelated in such a way that agents which affect either process can as a consequence thereby affect the other; absence of cell

division in itself will limit growth.[1,2] The question then arises as to what extent size differences between corresponding structures of gamma-plantlets (seedlings growing without cell division after 800 kr gamma irradiation of wheat grains[3,4]) and unirradiated controls are consequences of mitotic inhibition itself rather than consequences of other actions of radiation. Due to the complex interrelation between growth and cell division, this question can be answered only by studying structures that grow without cell division both in gamma-plantlets and in unirradiated controls. Such a study is summarized in this report.

Results. – During wheat germination the epiblast and coleorhiza grow without cell division, even in unirradiated plants, and therefore are growing, multicellular structures in which there can be no radiation-induced mitotic inhibition for the simple reason that there is no mitosis to inhibit.[5] This fact permits determination of the radiosensitivity of growth in a system uncomplicated by radiation-induced mitotic inhibition. Final size of either epiblast or coleorhiza is the same in gamma-plantlets and in unirradiated controls.

After division has ceased in epidermal cells of normally growing wheat roots, a root hair grows as a projection of an individual cell. Thus, the growth of root hairs provides another means of examining the radiosensitivity of growth of structures which develop without cell division in both irradiated and unirradiated plants. This instance is especially interesting because gamma-plantlet root hairs grow on an organ whose growth has already been decreased by radiation-induced mitotic inhibition. The gamma irradiation, which prevents detectable mitosis and limits growth of these roots, has no effect on the final size and form of root hairs.

Discussion. – These results with epiblast, coleorhiza, and root hairs, all of which grow without cell division during germination even in unirradiated plants, indicate surprisingly little radiosensitivity of growth that is uncomplicated by radiation-induced mitotic inhibition. These findings contrast sharply with the greatly reduced extents of growth in gamma-plantlet roots, leaves, and coleoptiles, which do grow with cell division during germination of unirradiated plants.[1,4] For the particular case of wheat germination in which our radiation treatments are given to the air-dry grains before sowing, these results can be summarized in the generalization: The ultimate extent of growth is reduced in those structures which suffer radiation-induced mitotic inhibition, but is not reduced in those structures which develop without cell division in unirradiated controls and therefore have no mitosis for the radiation to inhibit. This established generalization leads to the inference that in gamma-plantlets the smaller final sizes attained by organs that normally would have grown with cell division can be attributed to radiation-induced mitotic inhibition and not significantly to other possible effects of radiation. To the extent this inference may be correct, the final sizes actually attained by gamma-plantlets indicate the extent of growth theoretically possible without cell division under the conditions of the experiment. To the extent this inference may be incorrect, consideration of the extent of growth of gamma-plantlets would lead to an underestimation of the ultimate extents of growth theoretically possible had only cell division been affected by the irradiation. Thus, gamma-plantlets, in addition to giving an indication of the extent to which concurrent cell divisions are not essential for growth, also give an indication of the extent to which cell divisions are essential for sustaining normally continuing growth.

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[1] A. H. Haber, *Am. J. Botany* **49**, 583 (1962).

[2] A. H. Haber and D. E. Foard, *Am. J. Botany* **50**, 937 (1963).

[3] A. H. Haber, W. L. Carrier, and D. E. Foard, *Am. J. Botany* **48**, 431 (1961).

[4] D. E. Foard and A. H. Haber, *Am. J. Botany* **48**, 438 (1961).

[5] D. E. Foard and A. H. Haber, *Am. J. Botany* **49**, 520 (1962).

19.3 OPPOSITE EFFECTS OF GALACTURONIC ACID ON ONSET OF ELONGATION VS SUBSEQUENT ELONGATION IN SEMINAL ROOTS OF GERMINATING WHEAT

T. J. Long A. H. Haber

Introduction. – In seed germination the distinction between the onset of elongation and subsequent elongation is implied in the concept of dormancy, a condition which refers to the embryo as a whole. This report is concerned with an

embryo (wheat) with multiple root axes and deals with elongation of the lower lateral seminal roots *after* the primary root has already begun to grow. The results point to a distinction between emergence vs subsequent elongation of these wheat seminal roots which is analogous to the distinction between emergence vs subsequent elongation in seeds that have a single embryonic axis and are considered to exhibit dormancy (e.g., lettuce).

Results. – Embryos, not including scutella, were excised from wheat grains, planted on media containing various concentrations of glucose and galacturonic acid, and examined five days later after growth in darkness. There was no onset of seminal root elongation (by the criterion of emergence through the coleorhiza) without addition of glucose. All the primary roots grew on certain glucose concentrations that were limiting for subsequent primary root elongation and for percentage emergence of lower lateral seminal roots. Addition of galacturonic acid to these glucose concentrations increased the percentage emergence of the lower lateral seminal roots, but decreased the length per emerged lower lateral seminal root. That galacturonic acid truly retards elongation subsequent to emergence follows from two considerations: first, the frequency distributions of lengths among the dissimilar populations of emerged lower lateral seminal roots, and second, from the mean values and confidence limits of lengths for the comparable populations of primary roots.

The frequency distribution curves of the lower lateral seminal root lengths tend to be bimodal, with a mode at or near zero elongation representing the unemerged roots. This bimodal characteristic is, of course, either less pronounced or absent for those treatments in which most of the lower lateral roots emerge.

Discussion. – After elongation has already begun along one axis (the primary root) of the wheat embryo, the elongation along other axes (the lower lateral seminal roots) exhibits two formal similarities to the elongation in a seed exhibiting typical dormancy and having only a single embryonic axis (e.g., lettuce). These formal similarities are (1) a bimodal frequency distribution curve of elongation with one mode at or very near zero elongation and (2) certain instances in which chemical treatments that stimulate the initiation of elongation are inhibitory to subsequent elongation; this suggests that the mechanisms underlying the onset of growth are in one or more respects different from the mechanisms underlying subsequent growth. (Compare the opposite effects of galacturonic acid on initiation of elongation vs maintaining subsequent elongation, as described in this report, with the formally similar actions on lettuce seed germination of thiourea[1] and kinetin.[2]) These formal similarities indicate that the conceptual and statistical treatments used in interpreting elongation in seeds which, like lettuce, exhibit dormancy, must similarly be kept in mind when interpreting elongation of certain morphological axes of complex embryos even after other morphological axes have already begun their elongation. Thus, with respect to conceptual and statistical treatment of data, one should consider that in the germination of seeds with several embryonic axes the distinction between initiation of growth vs subsequent growth must be made *for each axis*, rather than for the embryo as a whole. With respect to the interrelation of cell division and expansion also, the initiation of growth in each of the organ primordia of the mature wheat embryo is analogous to the breaking of dormancy in a lettuce seed.[3] At present it is uncertain whether this expression of superficial similarities may also be an expression of fundamental similarities in physiological mechanisms.

[1] R. C. Thompson and W. F. Kosar, *Plant Physiol.* **14**, 567 (1939).

[2] C. O. Miller, *Plant Physiol.* **31**, 318 (1956).

[3] D. E. Foard and A. H. Haber, *Am. J. Botany* **48**, 438 (1961).

19.4 CELL DIVISION AND INITIATION OF LATERAL ROOT PRIMORDIA

D. E. Foard N. Ann Payne

Introduction. – From previous studies, the suggestion has been made that cell divisions do not play an essential role in the normal alterations of growth and its polarization involved in the

initiation of organ primordia.[1] In this study colchicine, which can prevent nuclear and cell division without preventing growth, was used to investigate the extent to which growth and morphogenesis can occur without cell division in the earliest detectable stages in the formation of lateral root primordia. Wheat was germinated in solutions of colchicine to prevent cell divisions without preventing growth and morphogenesis in the pericycle and endodermis, where lateral root primordia are initiated. (Initiation here implies nothing more than the occurrence of changes that permit the detection of an organ primordium where none was previously detectable.)

Results. – In the normal course of initiation of lateral (branch) roots, primordia originate entirely in the pericycle and endodermis (each of which in cross sections of wheat roots consists of only a single layer of cells) only between two successive protoxylem poles and facing a sieve tube. The bulk of a lateral root arises from the pericycle. In the pericycle the very earliest cell divisions concurrent with the initiation of lateral root primordia are oriented in a periclinal manner, that is, the new cell walls are parallel with the circumference of the main root and perpendicular to what will become the longitudinal axis of the lateral root. Within a localized region of the pericycle and endodermis, then, a new organ primordium is formed where none was previously detectable, and oriented cell divisions occur concurrently with the initiation of the new lateral root primordium.

When the wheat grain is sown on 0.03% colchicine, the embryo grows into a seedling usually having a root system of only three elongate roots, the main (primary) seminal root and the pair of lower seminal roots. Each of these three roots undergoes only limited growth and in the apical region forms the typical swelling, or "colchicine-tumor." Sections of such roots reveal that without any cell divisions in pericycle and endodermis after sowing, structures (here called "primordiomorphs") having the general shape of lateral root primordia, and thus quite distinct from "c-tumors," are formed in these tissues. The primordiomorphs originate only in those regions of the pericycle and endodermis between two successive protoxylem poles and facing a sieve tube, and thus have the same site of origin as normal lateral root primordia. Nuclei in the primordiomorphs are much larger than those in cells of the pericycle and endodermis that do not participate in the formation of such structures. In addition to identity of site of origin, primordiomorphs have other features of typical lateral root primordia. In summary, the similarities between primordiomorphs and typical lateral root primordia that develop with cell division are:

1. They originate only in the pericycle and endodermis and only between two successive protoxylem poles facing a sieve tube.

2. They stain intensely during early development.

3. They have a paraboloid-like shape.

4. They compress the cortical parenchyma during growth away from the vascular cylinder.

5. Interphase (including chromosome replication), prophase, and metaphase of mitosis occur in them.

Discussion. – We infer that the large nuclei present in the primordiomorphs are polyploid restitution nuclei, typically formed after chromosomal replication without subsequent nuclear division and cytokinesis, due to the inhibition of mitosis by colchicine ("c-mitosis").[2] The smaller, apparently normal, observed nuclear size in pericyclic and endodermal cells that do not participate in the formation of primordiomorphs is to be expected, since cells of the pericycle and endodermis that do not participate in the formation of lateral root primordia do not usually enter mitosis. Since there is a range of nuclear volumes among cells of primordiomorphs, we infer that during growth of primordiomorphs some cells repeatedly enter but do not complete mitosis. Thus in the primordiomorphs, although the later steps in mitosis are prevented, the first steps do indeed occur, sometimes more than once. The normalcy of these primordia, apart from the absence of cell division during their formation, depends upon the extent to which roots developing from them after release of the inhibition of cell division are normal. Such a study is in progress.

[1] A. H. Haber and D. E. Foard, *Am. J. Botany* **50**, 937 (1963).

[2] A. Levan, *Hereditas* **24**, 471 (1938).

19.5 PHOTOSYNTHESIS

W. A. Arnold J. B. Davidson[1]
W. F. Bertsch M. A. Kamrin
J. R. Azzi

Photosynthesis is the major process on earth by which organic material is synthesized from inorganic material. The chemical free energy required for this process is supplied by converting radiant energy from the sun into oxidizing and reducing power, so that water may be oxidized to oxygen, and carbon dioxide reduced to sugars. Work in this laboratory is aimed at understanding the mechanism of the energy conversion steps.

The delayed singlet emission from functionally active chlorophyll of living plants allows direct measurement of electron transitions within the photosynthetic apparatus. This delayed light emission at 1 msec after illumination appears to reflect energy conversion processes during the forward reactions, in agreement with the 10-msec turnover time of the photosynthetic unit (group of cooperating chlorophyll molecules).

We have been studying details of the emission spectrum at 1 msec in an attempt to determine whether the emission is from the bulk of the chlorophyll, or from a small component (or "reaction center") in the photosynthetic unit. We cannot answer this question until our studies of the effects of exciting wavelength and intensity, and of certain poisons, have been concluded.

The presence of more than one photoreaction in photosynthetic energy conversion has been deduced from many lines of evidence, including changes in the delayed light decay on excitation with different wavelengths. It has been suggested in the literature that the energies of two red quanta might combine to produce an excited triplet state of chlorophyll. If such a mechanism is actually present, then some blue fluorescence should be detectable even though the transition probability to the singlet would be very small. We determined that the intensity of blue fluorescence from living *Chlorella* is less than 10^{-8} of the red fluorescence. This indicates that an excited triplet of chlorophyll is probably not involved in the initial steps of photosynthesis.

An apparatus has been built for study of fluorescence from living plants under steady-state conditions of repetitive excitation. The buildup of fluorescence during each excitation period is approximately exponential, with a time constant the order of 50 msec. The decay of some fluorescence-inducing species is measured during the "dark" periods with use of a dim measuring beam to excite fluorescence. The "dark" decay is also nearly exponential, and has a time constant similar to that of the buildup which occurred during the excitation period. The temperature dependence of both processes suggests enzymatic reactions, and further studies are being carried out in the hope of following the fate of a long-lifetime intermediate which is closely associated with chlorophyll.

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20. Biophysics

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J. E. Donnellan
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R. A. McGrath
M. L. Randolph
R. B. Setlow[b]
Sohei Kondo[c]
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AEC-NASA Space Biology Program (20.1)

[a]Leave of Absence, USAEC, Division of Biology and Medicine, Washington.

[b]Dual assignment.

[c]Visiting investigator from abroad.

20.1 PRELIMINARY DOSIMETRY OF A P^{32} THIN-PLATE SOURCE FOR RADIATION–SPACE-FLIGHT INTERACTION EXPERIMENT[1]

Sohei Kondo J. L. Hosszu

A device to be used for irradiation of blood cells in a spacecraft should be as light as possible. Phosphorus-32 has been selected as one of the most suitable sources.

Four kinds of dosimetric data are relevant:

1. absolute absorbed dose,
2. relation of relative dose to depth, both in a tissue-like material,
3. distribution of P^{32} nuclei on the source plate,
4. intensity and energy spectrum of bremsstrahlung.

The source used was produced by the Isotopes Sales Department of ORNL and consisted of a thin platinum disk (0.16 g/cm^2 thick and *ca.* 4 cm in diameter) with *ca.* 2 mc of P^{32} deposited electrolytically. Using photographic films, the data of kinds 1 to 3 have been roughly estimated, and those of kind 4 have been rather carefully measured by a scintillation counter and a Victoreen ion chamber.

Film measurements revealed that the P^{32} distribution on the platinum disk is not uniform. Therefore the estimates of items 1 and 2 could only give the orders of magnitude, which agree with theory.

The bremsstrahlung intensity was *ca.* 0.5 mr/hr at 10 cm from the source placed inside the packaging container of 1.25-mm-thick aluminum. The bremsstrahlung spectrum measured with a 400-channel analyzer is of complicated nature, one peak (66.8 kev) corresponding to the characteristic x rays of the *K* shell of platinum and another peak (3.45 kev) caused by increase in absorption by aluminum with decrease in energy of bremsstrahlung.

Further dosimetry will be carried out with improved P^{32} sources by using various types of scintillation detectors and some other improved dosimeters.

[1]AEC-NASA Space Biology Program.

20.2 DECAY OF FREE RADICALS AND ENERGY BARRIERS IN THEIR RE-PAIRING

M. L. Randolph Mitzie G. Miller

Introduction. – The previously introduced study[1] on x-ray-induced free radicals in crystalline amino acids at various temperatures has been extended and refined. Basically our intent is to better understand the kinetics of re-pairing of unpaired electron spins – both on the qualitative basis of order of reaction kinetics and on the quantitative basis of energy barrier between the unpaired-electron and paired-electron states.

Results and Discussion. – A second graphical analysis of more extensive normalized data has been performed. For glycine the data are described by second-order decay kinetics for each of two components with different energies of re-pairing. Figure 20.2.1 gives the Arrhenius plot for glycine, and Table 20.2.1 the numerical values for all materials tested. Comparing with the work of Ghosh and Whiffen[2] we believe the slower-decaying radical is $^{+}H_3N\cdot\overset{\circ}{C}H\cdot CO_2^{-}$ and the faster-decaying radical $\overset{\circ}{N}H_2$. We have not noted the $\overset{\circ}{C}H(OH)\cdot CO_2H$ radical. In glutamic acid we find a stable radical species and an initially more abundant species which decays by second-order kinetics at and below 90°C. At 120°C, both decay in air by first-order kinetics, which we think may arise from interaction with O_2 when the material is at or near the sublimation point.[3] Alanine decay follows second-order kinetics from 23 to 150°C but probably has a second faster-decaying species which we have not analyzed. Glycyl glycine decays the least rapidly of these materials and seemingly has only one component. Leucine decays most rapidly, largely by interaction with air at 70°C. The order of its kinetics is not clearly established.

[1]M. L. Randolph and M. G. Miller, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 203.

[2]D. K. Ghosh and D. H. Whiffen, *J. Chem. Soc. (London)* **1960**, 1869–73.

[3]D. Gross and G. Grodsky, *J. Am. Chem. Soc.* **77**, 1678 (1955).

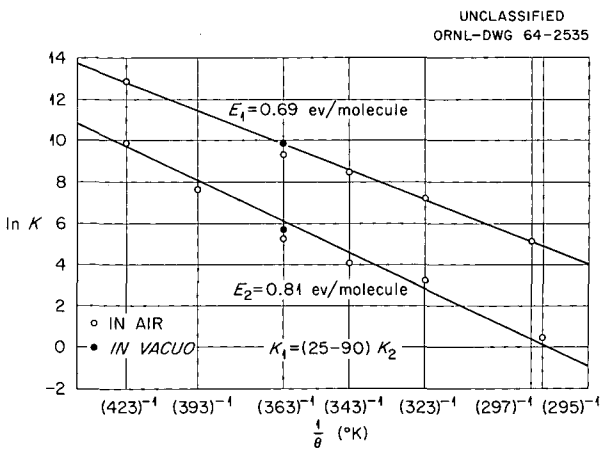

Fig. 20.2.1. Arrhenius Plots for Glycine.

Table 20.2.1. Results of Analyses of Free-Radical Decay Curves Taken at Various Temperatures

Material	Temperature (°C)	Approximate Relative Rate Constants at 23°C	Relative Initial Concentration	Energy: Electron Volts per Molecule	Energy: Kilocalories per Mole
Alanine	23–150	1		1.06	24.4
Glycine	23–150	1000	~3	0.69	15.9
		9	~1	0.81	18.6
Glutamic acid	23–90	40	~3	~0.8	~18
	23–90		~2	Stable	
	120–150			0.3?[a]	7?[a]
Glycyl glycine	23–90	7		~0.35	~8

[a]Radical species generated on heating; zero-order kinetics assumed.

20.3 PRODUCTION OF *BACILLUS MEGATERIUM* SPORES

J. E. Donnellan, Jr. E. F. Phares[1]

Introduction. – For many studies of biological systems it is desirable to have a stable supply of material that may be used for a series of experiments covering a long period of time. Dry bacterial spores may be readily adapted to this problem as they remain viable with no apparent change in properties for many years. With a view to studying the effect of irradiation on nucleic acid synthesis in bacterial spores, we have produced 74 g dry weight of *Bacillus megaterium* spores.

Results. – The method of Levinson and Sevag[2] was used with suitable alterations for adaptation to the pilot plant facility in the Biology Division. The medium contained 0.5% liver extract (Wilson and Co. "Liver Fraction B") in 10 m*M* potassium phosphate, pH 6.5. An inoculum of log phase *B. megaterium* cells[3] in the same media was used and the cells incubated at 30°C for 37 hr. At this time, 95 to 100% of the cells had formed spores of which about 80% were free of sporangia. The spores were harvested by centrifugation and washed three times with 10 m*M* potassium phosphate, pH 4.5, and at least six times with distilled water. During the washings, vegetative cells, debris, and spores in sporangia were removed to yield a product containing greater than 99% free spores. The washed spores were suspended in distilled water and lyophilized with a final yield of 74 g dry weight. The spores are stored at 4°C in vacuo over silica gel.

[1]Enzymology Group.

[2]H. S. Levinson and M. G. Sevag, *J. Gen. Physiol.* **36**, 617 (1953).

[3]Derived from a culture supplied by Dr. H. S. Levinson, U.S. Army Natick Laboratories, Natick, Mass.

20.4 INCREASED H^3-THYMIDINE INCORPORATION IN IRRADIATED SLIME MOLD

R. A. McGrath R. W. Williams

Introduction. – This work concerns the mechanisms involved in repair of radiation damage. Damage following exposure to x rays shows up in the slime mold, *Physarum polycephalum,* as mitotic delay (among other events). After one cycle of DNA synthesis, specific activity of the DNA is 30% greater than that of nonirradiated preparations. During cell cycles which follow the delayed mitosis, irradiated and nonirradiated preparations are indistinguishable.

Results and Conclusions. – A slime mold (*P. polycephalum*) which divides with mitotic synchrony was exposed to 25 kr of x rays and then placed in culture medium containing 1 μc/ml H^3-thymidine. The succeeding mitosis was delayed 8 to 10 hr. Four hours after division DNA was extracted and compared to DNA from nonirradiated cultures which had been in H^3-thymidine during one cycle of DNA synthesis. Specific activity of DNA from irradiated preparations was 30% greater than that from nonirradiated cultures. To determine whether a corresponding increase in DNA had occurred after irradiation, preparations were stained with the Feulgen reagent and the amount of dye bound per nucleus determined spectrophotometrically. The ratio of Feulgen-stain units (irradiated : nonirradiated) was 1 : 1. It is evident, then, that an increase in specific activity of DNA has occurred after irradiation without an increase in DNA.

Attention was focused on various ways in which additional tritium (presumably as H^3-thymine) could be bound to DNA. DNA was exposed to ultraviolet light (280 mμ) thereby forming thymine dimers (TT) between adjacent thymines. After hydrolysis, the ratio of radioactivity (thymine : TT) was determined. No differences were observed between irradiated and nonirradiated DNA's. This experiment rules out addition of chains of thymine (poly-T) to the DNA after irradiation. DNA was heated and rapidly cooled in 1% formaldehyde, then passed through Sephadex columns. No new peaks of radioactivity appeared in the effluent. Since Sephadex separates compounds on the basis of molecular size it is clear that additional small H^3-containing pieces are not hydrogen bonded to the main DNA structure.

Other obvious explanations can be proposed to explain the increase in specific activity without an increase in DNA: (1) Tritium in the pool of thymidine derivatives may be increased or (2) pieces of DNA can be removed from the main structure and be replaced by more heavily labeled pieces. Data are not yet sufficient to distinguish between these latter possibilities.

20.5 EVIDENCE FOR TWO MECHANISMS OF PHOTOREACTIVATION IN *ESCHERICHIA COLI* B

John Jagger R. S. Stafford

Introduction. – We have suggested that photoprotection from ultraviolet killing in *Escherichia coli* operates by inducing a growth-division delay after plating that permits more time for natural dark recovery mechanisms to operate.[1] If this idea is correct, then it should be possible to obtain recovery by inducing this delay immediately after ultraviolet irradiation, as well as before ultraviolet irradiation. The following report shows that this indeed can be done. This further supports our proposed mechanism of photoprotection. More important, however, it shows that what we normally call "photoreactivation" consists sometimes of two different processes, one the usual photoenzymatic process and the other a photochemical process similar to that involved in photoprotection.

Results. – Experiments were done with *E. coli* B (Harm) and with a mutant derived from it, *E. coli* B phr⁻, which does not show photoreactivation under certain conditions.[2] We found that the mutant can be photoreactivated if it is in the logarithmic phase of growth and if the wavelength is short enough. Figure 20.5.1 shows that both the mutant and the parent strain exhibit photoprotection, which normally occurs at 3341 A, but not at 4047 A.[3] Figure 20.5.2 shows that the parent

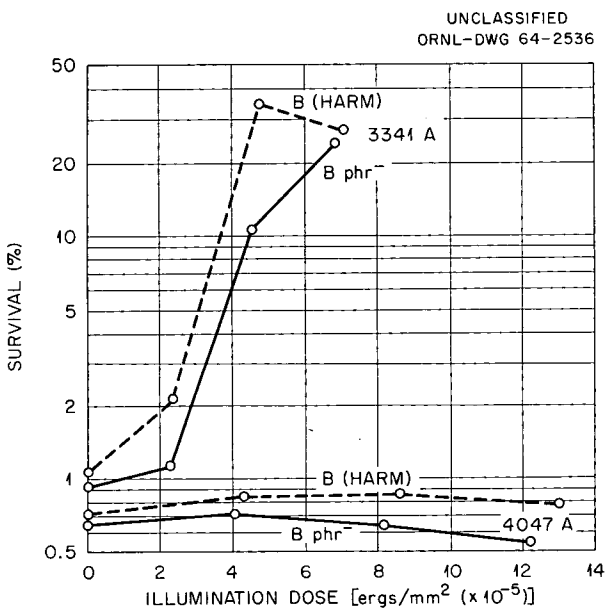

Fig. 20.5.1. Effect of Pre-Ultraviolet Illumination at 3341 and 4047 A on Survival of *E. coli* B (Harm) and *E. coli* B phr⁻ Irradiated with Ultraviolet (2537 A) to About 1% Dark Survival. Percent survival on a logarithmic scale is plotted against illumination dose. Dose rate of illumination was 250 ergs mm^{-2} sec^{-1}. Data are from a typical single experiment.

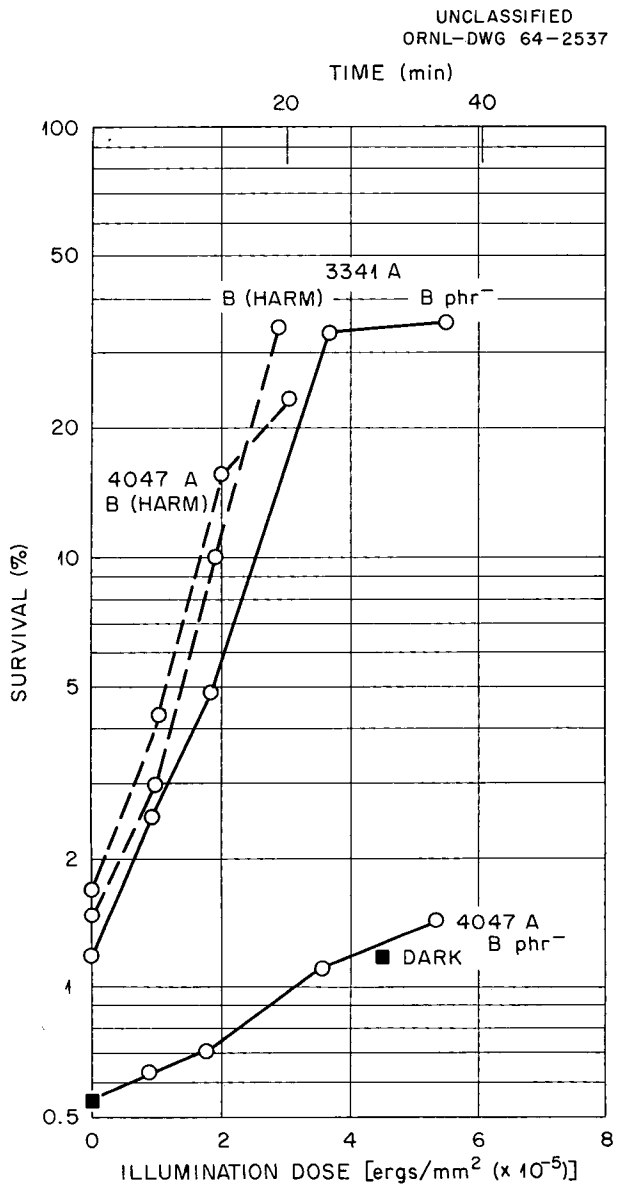

Fig. 20.5.2. Effect of Post-Ultraviolet Illumination. The square symbols represent liquid holding recovery in the dark over the time (upper abscissa) required to treat B phr⁻ at 4047 A. Otherwise similar to Fig. 20.5.1.

strain shows photoreactivation at both 3341 and 4047 A, as expected for enzymatic photoreactivation,[3] but that the mutant shows photoreactivation only at 3341 A. Thus, the action spectrum for photoreactivation of the mutant is similar to the action spectrum for photoprotection and quite dissimilar from the action spectrum for enzymatic photoreactivation.

We therefore supposed that the photoreactivation observed in the mutant was really a phenomenon similar to that of photoprotection. To test this idea, we studied the dependence of the amount of recovery per unit dose (recovery efficiency) on the dose rate of the light and upon the temperature during illumination.

Photoreactivation of the mutant showed no dependence on dose rate and practically no dependence upon temperature. Furthermore, illumination at 3341 A induces a large growth delay in the mutant, while illumination at 4047 A has no such effect. Therefore, by all these tests, photoreactivation of the phr$^-$ strain behaves in a fashion similar to that of photoprotection and different from that of photoreactivation.[4]

Photoreactivation of the parent strain shows a strong dependence upon dose rate and temperature at 4047 A, but only a slight dependence upon these factors at 3341 A. Again, 3341 A induces a large growth delay, while 4047 A does not. Thus, photoreactivation of the parent strain, *E. coli* B (Harm), behaves like enzymatic photoreactivation at 4047 A, but like photoprotection at 3341 A.

Conclusions. – There is no doubt that the mechanism of photoreactivation at 3341 A in log-phase *E. coli* B (Harm) is largely different from that at 4047 A. Thus, there are two mechanisms of photoreactivation in this strain.

By all the tests we have made so far, (1) the primary mechanism at 4047 A in the parent strain is similar to that of enzymatic photoreactivation, such as is displayed by in vitro enzymatic systems, and that involves direct repair of DNA, while (2) the primary mechanism at 3341 A is similar to that of photoprotection, a purely photochemical reaction that operates through the induction of a growth-division delay that in turn permits dark repair of DNA.

The mutant, phr$^-$, appears to display only the second type of photoreactivation, that which is similar in mechanism to photoprotection.

These experiments show that action spectra for photoreactivation, as well as studies of the dependence of photoreactivation on dose rate and temperature, must be reexamined in the light of possible interference by a purely photochemical reaction that probably bears no direct relation to the phenomenon of enzymatic photoreactivation.

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[1] J. Jagger, W. C. Wise, and R. S. Stafford. *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, pp. 200–201.

[2] W. Harm and B. Hillebrandt, *Photochem. Photobiol.* 1, 271 (1962).

[3] J. Jagger and R. S. Stafford, *Photochem. Photobiol.* 1, 245 (1962).

[4] J. Jagger, *Radiation Res.* **13**, 521 (1960).

20.6 ABSORBANCE CHANGES IN THIN THYMINE FILMS EXPOSED TO MONOCHROMATIC ULTRAVIOLET

Sohei Kondo R. B. Setlow

Recent evidence has pointed out the important role played by thymine dimers in the biological effects of ultraviolet. Previous workers have measured dimers in aqueous solvents. The present study of the mechanism of dimerization deals with solid films of thymine.

Results. – Thin films of thymine were deposited on quartz plates by a vacuum-sublimation method.[1] The absorption spectra of such films ($\lambda_{max} \sim 2700$ A) are qualitatively similar to but quantitatively different from those of aqueous solutions of thymine. Exposure of the films to 2800-A radiation results in an absorbance decrease at 2700 A and an increase at 2050 A. Further irradiation with 2380 A results in an increase in absorbance at 2700 A. The absorbance increase is about 30% of the decrease produced by the initial long-wavelength exposure. Similar results are obtained for a combination of 2537 and 2380 A. The absorbance changes with different irradiating wavelengths are qualitatively similar to those found for polynucleotides containing TT sequences and the magnitude of the reversible fraction is close

to the reported amount of dimers that can be detected chromatographically after dissolving irradiated solid thymine in water.[2] These results suggest that the reversible fraction represents dimers formed in the solid state.

[1]R. L. Sinsheimer, J. F. Scott, and J. R. Loofbourow, *J. Biol. Chem.* **187**, 299 (1950).

[2]K. C. Smith, *Photochem. Photobiol.* **2**, 503 (1963).

20.7 REMOVAL OF THYMINE DIMERS FROM BACTERIAL DNA

R. B. Setlow W. L. Carrier

Ultraviolet irradiation of DNA in vivo and in vitro forms dimers of adjacent thymines in DNA strands. The dimers are ultraviolet lesions of known structure and they act as blocks to DNA synthesis.[1,2] Radiation-resistant cells, such as *Escherichia coli* B/r, are able to recover the ability to make DNA after a lag period during which the dimers are eliminated from the bacterial DNA and appear in the acid-soluble fraction of cells.[3] Because dimer removal seems to be a necessary step in the recovery process, we have investigated several aspects of dimer elimination.

1. The pieces removed from DNA are small. Dimer-containing oligonucleotides in the acid-soluble fraction have chromatographic mobilities on DEAE paper that correspond to tri- and tetranucleotides that contain a dimer. The distribution of dimers among the oligonucleotides changes only slightly with time – a finding that indicates that these fragments are not the limit nuclease digests of large polynucleotides. Moreover we have been unable to find (by gel filtration of cell lysates with Sephadex column G-100) any dimer-containing polymers whose sizes are between 4 and 60 bases long.

2. Chemical interference with biological repair also interferes with dimer removal. Cells treated with acriflavin after ultraviolet show decreased colony survival and increased mutations.[4] We found that acriflavin (2.5 μg/ml) increases the lag period before DNA synthesis resumes by about tenfold and decreases the rate of dimer removal by the same factor.

3. Direct photoreactivation is not possible after dimers are removed from DNA. Illumination of cells with 405 mμ immediately following ultraviolet results in the disappearance of dimers (presumably by conversion to thymine) and an increase in survival. Dimers in vitro are only split rapidly if they are in native DNA.[5] Therefore it is reasonable that photoreactivating illumination of B/r has no effect on colony formation or dimers if the time interval after ultraviolet is sufficient to allow dimer removal. If dimers remain in DNA, as they do in the sensitive strain B_{s-1}, photoreactivation of both survival and dimers is possible even after 1 hr growth following ultraviolet.

4. There are differences among sensitive, normal, and resistant strains of *E. coli*. Sensitive strains cannot remove dimers from their DNA but both normal (strain B) and resistant strains can. Therefore the resistance of B/r is not the result of its having a more efficient "dimer-cutting" mechanism. In fact strain B removes dimers slightly faster than does B/r. Preliminary ultracentrifugal analyses of DNA that was extracted from bacteria that were recovering from the effects of ultraviolet indicate that there are more single-strand breaks in the DNA of B than in the DNA of B/r. Thus the difference between the two strains may be in their "patching" mechanisms.

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