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Progress

BIOLOGY DIVISION

SEMIANNUAL PROGRESS REPORT

FOR PERIOD ENDING FEBRUARY 15, 1965

OAK RIDGE NATIONAL LABORATORY
operated by
UNION CARBIDE CORPORATION
for the
U.S. ATOMIC ENERGY COMMISSION

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For Period Ending February 15, 1965

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OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee
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UNION CARBIDE CORPORATION
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U. S. ATOMIC ENERGY COMMISSION

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9.3 Biosatellite A Preparatory Experiments – **F. J. de Serres, B. B. Webber, R. C. von Borstel, Anna R. Whiting** 110

9.4 Spontaneous Mutation Frequencies in *Habrobracon* – **A. R. Whiting, R. C. von Borstel, Diane J. Goins, Martha J. Bond** 110

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Publications and Lectures

PUBLICATIONS

During this report period a total of 348 papers, abstracts, and book reviews by members of the Biology Division have been published or are in press. Listed below are the 92 published papers, reports, small conference booklets, and books edited by Division members for the current period. The 34 published abstracts, 3 published book reviews, and 219 items in press are not listed.

The proceedings of the Symposium, **Molecular Action of Mutagenic and Carcinogenic Agents,** held in April 1964 at Gatlinburg, were published as a supplement to the October 1964 *Journal of Cellular and Comparative Physiology*.

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Adler, H. I., and Alice A. Hardigree	The relation between *E. coli* B and the filament forming *E. coli* K-12 mutants	*Microbial Genet. Bull.* **21**, 3–4 (1964)
Allen, R. C.	Animal health programs, Biology Division, Oak Ridge National Laboratory	*Lab. Animal Care* **14**, 260–63 (1964)
Anderson, N. G.	New centrifuges for biological research	*New Scientist* **24**, 732–34 (1964)
Anderson, N. G., H. P. Barringer, E. F. Babelay, and W. D. Fisher	The B-IV zonal ultracentrifuge	*Life Sci.* **3**, 667–71 (1964)
Anderson, N. G., H. P. Barringer, C. E. Nunley, R. E. Canning, E. F. Babelay, C. L. Burger, and C. T. Rankin	The Joint National Institutes of Health–Atomic Energy Commission Zonal Centrifugation Project. I. The development of low-speed zonal rotors	ORNL-3613
Bailey, P. C., and Sheldon Wolff	A comparison of x-ray- and ultraviolet-induced aberrations in pollen tube chromosomes of Tradescantia. II. Influence of protein synthesis inhibitors	*Radiation Botany* **4**, 121–25 (1964)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Bender, M. A	Chromosome breakage *in vitro*	Pp. 87–107 in *Mammalian Cytogenetics and Related Problems in Radiobiology, Proceedings of a Symposium Held in Brazil, October, 1962* (ed. by C. Pavan, C. Chagas, O. Frota-Pessoa, and L. R. Caldas), Pergamon, Oxford, 1964
	Synergistic effect of zero-g and radiation on white blood cells: an experiment for the Gemini III manned space flight	ORNL-TM-940
Bender, M. A, and P. Carolyn Gooch	Persistent chromosome aberrations in irradiated human subjects. II. Three and one-half year investigation	P. 457 in *The Year Book of Cancer* (1963–1964 Year Book Series) (ed. by R. L. Clark and R. W. Cumley), Year Book Medical Publishers, Inc., Chicago, 1964
Bennett, Michael, G. M. Shearer, A. C. Upton, and Gustavo Cudkowicz	Factors affecting the expansion of the stem cell pool in transplanted mouse bone marrow	*Nature* **204**, 351–53 (1964)
Berger, J. D., and R. F. Kimball	Specific incorporation of precursors into DNA by feeding labeled bacteria to *Paramecium aurelia*	*J. Protozool.* **11**, 534–37 (1964)
Brewen, J. G.	Kinetics of x-ray-induced chromatid aberrations in *Vicia faba* and studies relating aberration frequencies to the cell cycle	*Mutation Res.* **1**, 400–408 (1964)
Cameron, I. L., and R. E. Smith	Cytological responses of brown fat tissue in cold-exposed rats	*J. Cell Biol.* **23**, 89–100 (1964)
Cattanach, B. M.	Autosomal trisomy in the mouse	*Cytogenetics* **3**, 159–66 (1964)
Christenberry, K. W., A. C. Upton, and J. W. Conklin	Recording lens opacities	*Arch. Ophthalmol.* **72**, 667–69 (1964)
Chu, E. H. Y.	Abnormalities of sex chromosomes	Pp. 209–32 in *Mammalian Cytogenetics and Related Problems in Radiobiology, Proceedings of a Symposium Held in Brazil, October, 1962* (ed. by C. Pavan, C. Chagas, O. Frota-Pessoa, and L. R. Caldas), Pergamon, Oxford, 1964
Clayton, R. K., and W. F. Bertsch	Absence of delayed light emission in the millisecond time range from a mutant of *Rhodopseudomonas spheroides* which lacks functioning photosynthetic reaction centers	*Biochem. Biophys. Res. Commun.* **18**, 415–19 (1965)
Cleffmann, Günter	Function-specific changes in the metabolism of agouti pigment cells	*Exptl. Cell Res.* **35**, 590–600 (1964)
Cohn, W. E (coeditor)	*Progress in Nucleic Acid Research*, vol. 3 (363 pp.)	Ed. by J. N. Davidson and W. E Cohn, Academic, New York, 1964
Congdon, C. C	The early histologic effects of antigenic stimulation	*Arch. Pathol.* **78**, 83–96 (1964)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Conklin, J. W., A. C. Upton, and K. W. Christenberry	Further observations on late somatic effects of radiomimetic chemicals and x-rays in mice	*Cancer Res.* **25**, 20–28(1965)
Cosgrove, G. E., T. P. O'Farrell, S. V. Kaye, and P. B. Dunaway	Infection of laboratory-reared and wild cotton rats with cat tapeworm larvae	*J. Tenn. Acad. Sci.* **39**, 140–41 (1964)
Cudkowicz, Gustavo, and J. H. Stimpfling	Induction of immunity and of unresponsiveness to parental marrow grafts in adult F_1 hybrid mice	*Nature* **204**, 450–53 (1964)
Curtiss, Roy, III	Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*	*J. Bacteriol.* **89**, 28–40 (1965)
Davidson, Douglas	RNA synthesis in roots of *Vicia faba*	*Exptl. Cell Res.* **35**, 317–25 (1964)
Dekaban, A. S., M. A Bender, and G. E. Economos	Chromosome studies in mongoloids and their families	*Cytogenetics* **2**, 61–75 (1963)
de Serres, F. J.	Mutagenesis and chromosome structure	*J. Cellular Comp. Physiol.* **64** (suppl. 1), 33–42 (1964)
Dolin, M. I., and R. H. Baum	Evidence for thioether formation between 2-solanesyl-1,4-naphthoquinone and 2-mercaptoethanol	*Biochem. Biophys. Res. Commun.* **18**, 202–5 (1965)
Dolin, M. I., and K. B. Jacobson	Carbonyl addition to nicotinamide adenine dinucleotide in frozen solution. Spectral, fluorometric, and optical rotary properties of the oxidized acetone adduct	*J. Biol. Chem.* **239**, 3007–16 (1964)
Dulbecco, Renato	Mutagenic and carcinogenic agents (meeting report)	*Science* **145**, 300 (1964)
Ehling, U. H.	Strain variation in reproductive capacity and radiation response of female mice	*Radiation Res.* **23**, 603–10 (1964)
Feinstein, R. N., J. E. Seaholm, Judith B. Howard, and W. L. Russell	Acatalasemic mice	*Proc. Natl. Acad. Sci. U.S.* **52**, 661–62 (1964)
Fisher, W. D., G. B. Cline, and N. G. Anderson	Density gradient centrifugation in angle-head rotors	*Anal. Biochem.* **9**, 477–82 (1964)
Gengozian, Nazareth, and Gino Doria	Allotypic specificities in four strains of inbred mice as revealed by reciprocal immunizations	*J. Immunol.* **93**, 426–32 (1964)
Greenman, D. L., F. T. Kenney, and W. D. Wicks	On equating low molecular weight RNA with transfer RNA	*Biochem. Biophys. Res. Commun.* **17**, 449–53 (1964)
Grell, Rhoda F.	Distributive pairing: the size-dependent mechanism for regular segregation of the fourth chromosomes in *Drosophila melanogaster*	*Proc. Natl. Acad. Sci. U.S.* **52**, 226–32 (1964)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Hollaender, Alexander	Foreword	P. vii in *Mammalian Cytogenetics and Related Problems in Radiobiology, Proceedings of a Symposium Held in Brazil, October 1962* (ed. by C. Pavan, C. Chagas, O. Frota-Pessoa, and L. R. Caldas), Pergamon, Oxford, 1964
	Symposium on Molecular Action of Mutagenic and Carcinogenic Agents – Introduction	*J. Cellular Comp. Physiol.* **64** (suppl. 1) (1964) (191 pp.)
Hollaender, Alexander, and S. F. Carson	Biology Division semiannual progress report for period ending August 15, 1964	ORNL-3700
Jacobson, K. B., Susumu Nishimura, W. E. Barnett, R. J. Mans, Piero Cammarano, and G. D. Novelli	On the lack of a uniform specificity of aminoacyl ribonucleic acid synthetases from different organisms	*Biochim. Biophys. Acta* **91**, 305–12 (1964)
Jagger, John	Photoprotection from far ultraviolet effects in cells	Pp. 584–601 in *Advances in Chemical Physics*, vol. VII – *The Structure and Properties of Biomolecules and Biological Systems* (series ed. by I. Prigogine; vol. ed. by J. Duchesne), Interscience, division of Wiley, London, 1964
Jagger, John, and R. S. Stafford	Evidence for two mechanisms of photoreactivation in *Escherichia coli* B	*Biophys. J.* **5**, 75–88 (1965)
Karasaki, Shuichi	An electron microscopic study of lens regeneration in the adult newt	*J. Ultrastruct. Res.* **11**, 246–73 (1964)
Kimball, R. F.	The distribution of x-ray induced mutations to chromosomal strands in *Paramecium aurelia*	*Mutation Res.* **1**, 129–38 (1964)
	Physiological genetics of the ciliates	Pp. 243–75 in *Biochemistry and Physiology of Protozoa, vol. III* (ed. by S. H. Hunter), Academic, New York, 1964
King, Vera M., and G. E. Cosgrove	Intestinal helminths in various strains of laboratory mice	Pp. 375–76 in *The Year Book of Veterinary Medicine, vol. 2* – 1964 (ed. by W. F. Riley, Jr., K. W. Smith, and R. J. Flynn), Year Book Medical Publishers, Inc., Chicago, 1964
Kondo, Sohei	Variation in mutagenicity and radiation resistance with genome complexity and evolution	*Japan. J. Genetics* **39**, 176–98 (1964)
Lindsley, D. L., and L. Sandler	Meiotic behavior of tandem metacentric compound X chromosomes in *Drosophila melanogaster*	*Genetics* **51**, 223–45 (1965)
Magni, G. E., R. C. von Borstel, and S. Sora	Mutagenic action during meiosis and antimutagenic action during mitosis by 5-aminoacridine in yeast	*Mutation Res.* **1**, 227–30 (1964)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Makinodan, Takashi	In memory of Professor Harold R. Wolfe	Pp. v–vi in *The Thymus in Immunobiology: Structure, Function, and Role in Disease* (ed. by R. A. Good and Ann. E. Gabrielson), Hoeber Medical Division, Harper & Row, New York, 1964
Makinodan, Takashi, and W. J. Peterson	Growth and senescence of the primary antibody-forming potential of the spleen	*J. Immunol.* **93**, 886–96 (1964)
Mans, R. J., and G. D. Novelli	Ribonucleotide incorporation by a soluble enzyme from maize	*Biochim. Biophys. Acta* **91**, 186–88 (1964)
McArthur, W. H., C. C Congdon, and A. L. Kretchmar	Nitrogen balance in radiation chimeras	*Proc. Soc. Exptl. Biol. Med.* **117**, 171–75 (1964)
Mead, C. G.	The enzymatic condensation of oligodeoxyribonucleotides with polydeoxyribonucleotides	*Proc. Natl. Acad. Sci. U.S.* **52**, 1482–88 (1964)
Miller, O. L., Jr., G. E. Stone, and D. M. Prescott	Autoradiography of soluble materials	*J. Cell Biol.* **23**, 654–58 (1965)
Odell, T. T., Jr.	Studies on production and regulation of rat blood platelets	Pp. 389–92 in *Proceedings of the IX Congress of the International Society of Hematology, vol. II*, Universidad Nacional Autónoma de México, 1964
Odell, T. T., Jr., and T. P. McDonald	Methods for the estimation of the life span of platelets	Pp. 68–73 in *Blood Coagulation, Hemorrhage and Thrombosis – Methods of Study* (ed. by L. M. Tocantins and L. A. Kazal), Grune & Stratton, New York, 1964
Odell, T. T., Jr., T. P. McDonald, and F. L. Howsden	Native and foreign stimulators of platelet production	*J. Lab. Clin. Med.* **64**, 418–24 (1964)
Padilla, G. M., and I. L. Cameron	Synchronization of cell division in *Tetrahymena pyriformis* by a repetitive temperature cycle	*J. Cellular Comp. Physiol.* 303–7 (1964)
Padilla, G. M., I. L. Cameron, and G. L. Whitson	Cell Synchrony (meeting report of conference held at the Biology Division, Oak Ridge National Laboratory, October 9–10, 1964)	*Science* **147**, 175–77 (1965)
Pfuderer, Peter, P. Cammarano, D. R. Holladay, and G. D. Novelli	A helical model for the polysome in solution	*Biochem. Biophys. Res. Commun.* **18**, 355–58 (1965)
Popp, R. A., and W. St. Amand	A sex difference in recombination frequency in the albinism-hemoglobin interval of linkage group I in the mouse	*J. Heredity* **55**, 101–3 (1964)
Prescott, D. M.	The normal cell cycle	Pp. 71–97 in *Synchrony in Cell Division and Growth* (ed. by Erik Zeuthen), Interscience, division of Wiley, New York, 1964

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Ratty, F. J., and D. L. Lindsley	vg^U: *Ultravestigial*	*Drosophila Information Service* **38**, 30 (1963)
Russell, Diane B.	Experimental studies on mammalian chromosome aberrations	Pp. 61–86 in *Mammalian Cytogenetics and Related Problems in Radiobiology, Proceedings of a Symposium Held in Brazil, October, 1962* (ed. by C. Pavan, C. Chagas, O. Frota-Pessoa, and L. R. Caldas), Pergamon, Oxford, 1964
	Genetic and functional mosaicism in the mouse	Pp. 153–81 in *Role of Chromosomes in Development*, Academic, New York, 1964
Russell, W. L.	Studies in mammalian radiation genetics	*Nucleonics* **23**, 53–56, 62 (1965)
Sado, Toshihiko, and Takashi Makinodan	The cell cycle of blast cells involved in secondary antibody response	*J. Immunol.* **93**, 696–700 (1964)
Schuel, Herbert, and Regina Schuel	The induction of macro-molecular aggregation reactions and acid production in rat liver homogenates by calcium and magnesium ions	*J. Cellular Comp. Physiol.* **64**, 33–40 (1964)
Schuel, Herbert, S. R. Tipton, and N. G. Anderson	Studies on isolated cell components. XVII. The distribution of cytochrome oxidase activity in rat liver brei fractionated in the zonal ultracentrifuge	*J. Cell Biol.* **22**, 317–26 (1964)
Schwartz, Drew	Genetic studies on mutant enzymes in maize. V. *In vitro* interconversion of allelic isozymes	*Proc. Natl. Acad. Sci. U.S.* **52**, 222–26 (1964)
	Tissue specificity and the control of gene action	Pp. 201–3 in *Structure and Function of the Genetic Material, Erwin-Baur Memorial Lectures III, 1963*, Akademie-Verlag, Berlin, 1964
Setlow, Jane K., and D. E. Duggan	The resistance of *Micrococcus radiodurans* to ultraviolet radiation. I. Ultraviolet-induced lesions in the cell's DNA	*Biochim. Biophys. Acta* **87**, 664–68 (1964)
Setlow, R. B.	Molecular changes responsible for ultraviolet inactivation of the biological activity of DNA	Pp. 291–307 in *Mammalian Cytogenetics and Related Problems in Radiobiology, Proceedings of a Symposium Held in Brazil, October, 1962* (ed. by C. Pavan, C. Chagas, O. Frota-Pessoa, and L. R. Caldas), Pergamon, Oxford, 1964
	Physical changes and mutagenesis	*J. Cellular Comp. Physiol.* **64** (suppl. 1), 51–68 (1964)
Setlow, R. B., W. L. Carrier, and F. J. Bollum	Nuclease-resistant sequences in ultraviolet-irradiated deoxyribonucleic acid	*Biochim. Biophys. Acta* **91**, 446–61 (1964)
Stahl, Frank, and C. M. Steinberg	The theory of formal phage genetics for circular maps	*Genetics* **50**, 531–38 (1964)
Stapleton, G. E.	The ORNL space biology program, annual report, period ending June 30, 1964	ORNL-TM-924
Swartzendruber, D. C.	Phagocytized plasma cells in mouse spleen observed by light and electron microscopy	*Blood* **24**, 432–42 (1964)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Swenson, P. A., and R. B. Setlow	β-Galactosidase: inactivation of its messenger RNA by ultraviolet irradiation	*Science* **146**, 791–94 (1964)
Tyndall, R. L., K. B. Jacobson, and Ernestine Teeter	Ribonucleic acid fingerprints of virus-infected and uninfected HeLa cells	*Biochim. Biophys. Acta* **87**, 335–38 (1964)
Upton, A. C.	Cancer research 1964: thoughts on the contributions of radiation biology	*Cancer Res.* **24**, 1861–68 (1964)
Upton, A. C., M. L. Randolph, E. B. Darden, Jr., and J. W. Conklin	Relative biological effectiveness of fast neutrons for late somatic effects in mice	Pp. 337–45 in *Biological Effects of Neutron and Proton Irradiations, vol. II, Proceedings of the Symposium on Biological Effects of Neutron Irradiations, Held by the International Atomic Energy Agency at the Brookhaven National Laboratory, Upton, New York, 7–11 October 1963*, IAEA, Vienna, Austria, 1964
Van Dreal, P. A., and G. M. Padilla	CO_2 and cytokinesis in temperature-synchronized *Astasia longa*	*Biochim. Biophys. Acta* **93**, 668–70 (1964)
Walburg, H. E., Jr., A. C. Upton, R. L. Tyndall, W. W. Harris, and G. E. Cosgrove	Preliminary observations on spontaneous and radiation-induced leukemia in germfree mice	*Proc. Soc. Exptl. Biol. Med.* **118**, 11–14 (1965)
Webber, B. B.	Genetical and biochemical studies of histidine-requiring mutants of *Neurospora crassa*. III. Correspondence between biochemical characteristics and complementation map position of *hist-3* mutants.	*Genetics* **51**, 263–73 (1965)
	Genetical and biochemical studies of histidine-requiring mutants of *Neurospora crassa*. IV. Linkage relationships of *hist-3* mutants	*Genetics* **51**, 275–83 (1965)
Welshons, W. J.	Detection and use of cytological anomalies in the mouse	Pp. 233–43 in *Mammalian Cytogenetics and Related Problems in Radiobiology, Proceedings of a Symposium Held in Brazil, October, 1962* (ed. by C. Pavan, C. Chagas, O. Frota-Pessoa, and L. R. Caldas), Pergamon, Oxford, 1964
Williams, G. R., and G. D. Novelli	Stimulation of an *in vitro* amino acid incorporating system by illumination of dark-grown plants	*Biochem. Biophys. Res. Commun.* **17**, 23–27 (1964)
Yamada, Tuneo, and Marion E. Roesel	Effects of actinomycin D on the lens regenerating system	*J. Embryol. Exptl. Morphol.* **12**, 713–25 (1964)
(Members of the Biology Division)	Experimental Hematology – summary of reports of bone marrow conferences organized by members of the Biology Division, ORNL	Reprint No. 2935
(Members of the Biology Division)	Report of Conference on Cell Synchrony – program and abstracts of conference held October 9–10, 1964, at the Biology Division, ORNL (G. M. Padilla, chairman)	Small Conference Booklet No. 11

LECTURES

During the period August 16, 1964, to February 15, 1965, members of the Biology Division presented 242 lectures and speeches. This figure compares with 211 for the corresponding period of 1963–1964, and with 219 for the February 16–August 15, 1964, reporting period. Of the 242 lectures and speeches, a total of 95 was given at meetings of professional societies, and 116 on invitation of universities, laboratories, and scientific institutions in the United States and abroad. A total of 31 was presented as part of the Traveling Lecture Program.

For details on speaking activities outside the continental United States, see the section "Foreign Travel." Educational activities are described in the section by the same name (see Contents).

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I.	Genetic control of radiation sensitivity	1. Palo Alto Medical Research Foundation, Palo Alto, Calif. 2. Reed College, Portland, Ore. 3. University of California, Berkeley
	Radiation sensitivity of filament forming mutants of *Escherichia coli*	Pennsylvania State University, University Park
Adler, H. I. (and Alice A. Hardigree)	The growth and division of irradiated bacteria	Am. Soc. Microbiol., Kentucky-Tennessee Section, Atlanta, Ga.
Allen, R. C. (and Dorothy J. Moore)	Sex hormone-dependent esterase isozymes in mouse plasma [*Am. Zool.* 4, 394 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Anderson, N. G.	The development of zonal centrifuges	1. Ciba Foundation, London 2. Carlsberg Foundation, Copenhagen, Denmark 3. Karolinska Institutet, Stockholm 4. Uppsala University, Uppsala, Sweden 5. Institut de Recherches sur le Cancer, Villejuif (Seine), France 6. University of Glasgow, Glasgow, Scotland 7. Cambridge University, Cambridge, England
	Zonal centrifugation: an approach to the intracellular distribution of drugs	Food and Drug Administration Scientific Seminar Group, Washington, D. C.
	The zonal centrifuge	Navy Nuclear Sciences Seminar, Oak Ridge, Tenn.
Anderson, N. G. (and A. A. Barber)	Isolation of native glycogen by combined rate-zonal and isopycnic centrifugation	Biophysical Society, 9th Annual Meeting, San Francisco

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Barnett, W. E.	Coding properties of sRNA's	1. Duke University, Durham, N. C. 2. Florida State University, Tallahassee 3. Rice University, Houston, Tex. 4. University of Kentucky Medical School, Lexington 5. University of Texas M. D. Anderson Hospital and Tumor Institute, Houston 6. University of Georgia, Athens
	Coding properties of transfer RNA's	University of Florida Medical School, Gainesville
	Gene action	University of Florida Medical School, Gainesville
	Gene regulation	University of Florida Medical School, Gainesville
	The roles of RNA in protein synthesis	1. Florida Southern College, Lakeland 2. Mary Hardin-Baylor College, Belton, Tex. 3. Texas A & I College, Kingsville 4. Texas A & M University, College Station
Bell, Sandra L. (and Sheldon Wolff)	Effects of FUdR on DNA synthesis in *Vicia faba* [*J. Cell Biol.* **23**, 9A (1964)]	Am. Soc. Cell Biol., Cleveland
Bender, M. A	Cytogenetic effects of radiation in man	Eighth International Congress of Internal Medicine, Buenos Aires
	An experiment to measure human chromosome aberration rates from irradiation in the absence of gravity	Mammalian Cytology and Somatic Cell Genetics Conference, San Juan, Puerto Rico
Bender, M. A (and H. F. Smith, Jr.)	Radiation—zero G interaction experiment for the Gemini-III mission	Second Symposium on Protection Against Radiations in Space, Gatlinburg, Tenn.
Bollum, F. J.	Biological properties of polydeoxynucleotides	Albert Einstein School of Medicine, Yeshiva University, New York
	Biosynthetic polydeoxynucleotides	1. State University of New York, Buffalo 2. University of Kentucky Medical Center, Lexington
	New dimensions in polydeoxynucleotide synthesis	Biophysical Society, 9th Annual Meeting, San Francisco
Brewen, J. G.	Chromosomal aberrations induced in human leukocytes by cytosine arabinoside in relation to the cell cycle	Mammalian Cytology and Somatic Cell Genetics Conference, San Juan, Puerto Rico
	Radiation-induced chromosomal aberrations and their relation to the cell cycle	Johns Hopkins University, Baltimore, Md.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Burger, C. L. (and N. G. Anderson)	Acid soluble constituents, DNA content and free amino acids of irradiated and normal rat tissues	Biophysical Society, 9th Annual Meeting, San Francisco
Cameron, I. L.	The cell cycle	State University of New York, Syracuse
	The cell cycle and its chemistry	1. Knoxville College, Knoxville, Tenn. 2. Midwestern University, Wichita Falls, Tex. 3. Southwestern State College, Weatherford, Okla. 4. Virginia Polytechnic Institute, Blacksburg
	Cellular control of DNA synthesis	1. Southwestern State College, Weatherford, Okla. 2. Vanderbilt University, Nashville, Tenn.
	Specific nutritional synchronization of *Tetrahymena pyriformis*	Cell Synchrony Conference, Oak Ridge, Tenn.
Cameron, I. L. (and W. D. Fisher)	The separation of acid soluble nucleotides and related compounds from *Tetrahymena* during different growth conditions [*Am. Zool.* **4**, 408 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Cameron, I. L. (and E. E. Guile, Jr.)	A pattern of structural and biochemical changes during unbalanced growth of *Tetrahymena pyriformis* [*J. Cell Biol.* **23**, 17A (1964)]	Am. Soc. Cell Biol., Cleveland
Cameron, I. L. (G. M. Padilla and Betty M. Wysinger)	Metabolic regulation of the DNA-synthetic period in *Tetrahymena*	Biophysical Society, 9th Annual Meeting, San Francisco
Canning, R. E. (and N. G. Anderson)	Separation of subcellular fractions with a new zonal rotor	AIBS (Am. Soc. Zool.), Boulder, Colo.
Caro, L. G.	High resolution autoradiography	Southeast Electron Microscopy Society, Atlanta, Ga.
	Mechanisms of DNA transfer in bacterial mating	1. Biophysical Society, 9th Annual Meeting, San Francisco 2. Brandeis University, Waltham, Mass. 3. Cold Spring Harbor Biological Laboratory, Cold Spring Harbor, L. I., N. Y. 4. Massachusetts Institute of Technology, Cambridge 5. University of Colorado, Denver
	Protein synthesis, storage and discharge in the pancreatic acinar cell	University of Colorado, Denver
	Two applications of autoradiography to biological problems	XIth International Congr. of Cell Biol., Providence, R. I.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Charamella, L. J. (Roy Curtiss III, Paula E. Harris, and Claire M. Berg)	D-Cycloserine inhibition and resistance in *Escherichia coli* K-12	Am. Soc. Microbiol., Kentucky-Tennessee Section, Atlanta, Ga.
Chu, E. H. Y.	Induction of mammalian chromosome aberrations by ultraviolet light	1. Academia Sinica, Taiwan 2. Osaka University, Osaka, Japan 3. Zoological Institute, Hokkaido, Japan
	Mammalian chromosome cytology	National Taiwan University, Taiwan
	Radiation effect on mammalian cells and human cytogenetics	University of Kyoto School of Medicine, Kyoto, Japan
	Variations in the frequency of chromatid aberrations in mammalian cells induced by x-rays and ultraviolet light during different stages of the cell cycle	Mammalian Cytology and Somatic Cell Genetics Conference, San Juan, Puerto Rico
Congdon, C. C. (M. A. Kastenbaum and D. A. Gardiner	The extreme variation in mortality from secondary disease in radiation chimeras	Xth Congress of International Society of Haematology, Stockholm
	Factors affecting mortality from secondary disease	Radiation Protection and Recovery Conference, Houston, Tex.
Cosgrove, G. E.	Delayed somatic effects in mammals	Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn.
	Experimental radiobiology using laboratory animals	American Veterinary Radiology Section, Elkhart, Ind.
	Mammalian radiobiology (four lectures)	Wayne State University, Detroit, Mich.
	Radiation hazards to man	U. S. Navy Nuclear Sciences Seminar, Oak Ridge, Tenn.
Cudkowicz, Gustavo	Genetic interaction in the inheritance of mouse histocompatibility factors	1. University of Pavia, Pavia, Italy 2. Weizmann Institute of Science, Rehovoth, Israel
	Hybrid resistance to parental hemopoietic cell grafts: implications for bone marrow chimeras	Colloque du Centre National de la Recherche Scientifique on Transplantation of Allogeneic Hemopoietic Cells, Paris
Cudkowicz, Gustavo (and J. H. Stimpfling)	Lack of expression of parental isoantigen(s) in F_1 hybrid mice	1. The Hebrew University, Jerusalem, Israel 2. Xth Congress of the International Society of Blood Transfusion, Stockholm
Cudkowicz, Gustavo (Michael Bennett, G. M. Shearer, and A. C. Upton)	Functional characterization of the mouse marrow lymphocyte	Xth Congress of the International Society of Haematology, Stockholm
Curtiss, Roy, III	Genetic transfer by conjugation in *Escherichia coli*	1. Louisiana State University, Baton Rouge 2. St. Louis University, St. Louis 3. University of Kentucky, Lexington

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Curtiss, Roy, III (and L. J. Charamella)	The pro-lac region of the *Escherichia coli* K-12 genome	Am. Soc. Microbiol., Kentucky-Tennessee Section, Atlanta, Ga.
de Serres, F. J.	Impaired complementation between nonallelic mutations in Neurospora	Symposium on Genes and Chromosomes, Buenos Aires
Doherty, D. G.	Chemical protection against ionizing radiation	Mallinckrodt Chemical Works, St. Louis
	Enzymes, the biological catalysts	Winthrop College, Rock Hill, S. C.
Donnellan, J. E., Jr. (E. H. Nags and H. S. Levinson)	Nucleic acid synthesis during germination and postgerminative development	Third Spore Conference, Allerton Park, Ill.
Donnellan, J. E., Jr. (and R. B. Setlow)	Uv photoproducts in bacterial spores	Biophysical Society, 9th Annual Meeting, San Francisco
Finamore, F. J.	Oligonucleotides in embryonic development	1. University of Georgia, Athens 2. University of Miami, Coral Gables, Fla. 3. University of Puerto Rico, Mayaguez 4. University of Puerto Rico, Rio Piedras
	Unique pyrophosphate ester in brine shrimp	1. University of Puerto Rico, Mayaguez 2. University of Puerto Rico, Rio Piedras
	Oligonucleotides-RNA polymerase	University of Puerto Rico, Rio Piedras
Füchtbauer, Walter	Action of ionizing radiation on photosynthetic reactions of isolated chloroplasts	University of Utah, Salt Lake City
Gooch, P. Carolyn (and M. A Bender)	Chromosome aberrations induced by neutron irradiation of human leukocytes	Third Mammalian Cytology and Somatic Cell Genetics Conference, San Juan, Puerto Rico
Goodman, Joan Wright	Concerning the capacity of lymphocytes to give rise to erythropoiesis	Colloque du Centre National de la Recherche Scientifique on Transplantation of Allogeneic Hemopoietic Cells, Paris
	Differences in the specific immunological nonresponsiveness between genetically tolerant mice and those made tolerant by inoculation at birth	Xth Congress of the International Society of Blood Transfusion, Stockholm
Grell, E. H.	The genetic control of some enzymes in Drosophila	University of Buenos Aires, Buenos Aires
Grell, Rhoda F.	Chromosome pairing, crossing over, and segregation in *Drosophila melanogaster*	Symposium on Genes and Chromosomes, Buenos Aires

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Gude, W. D.	Biological effects of radiation	Randolph-Macon Woman's College, Lynchburg, Va.
	Biological effects of radiation on mammals	1. Powell High School, Powell, Tenn. (three lectures) 2. Lenoir City High School, Lenoir City, Tenn. (four lectures)
Haber, A. H.	Cellular organization and differentiation	University of Tennessee, Knoxville
Hanna, M. G., Jr.	Germinal center changes and plasma cell reaction during the primary immune response [*Am. Zool.* **4**, 409 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Hanna, M. G., Jr. (and C. J. Wust)	Actinomycin D effect on the primary immune response in mice	Radiation Protection and Recovery Conference, Houston, Tex.
Harrison, A. P., Jr.	Uses of microorganisms in cancer research	Am. Soc. Microbiol., South Central Branch, New Orleans
Heddle, J. A.	Chromosome aberrations in barley induced by neutrons and x-rays	University of Tennessee, Knoxville
Heddle, Judith G.	Teaching and research in nutrition (two lectures)	University of Tennessee, Knoxville
Heddle, Judith G. (R. A. Popp, R. E. Canning, and R. C. Allen)	Isolation of albumin and albumin esterase in mouse serum [*Am. Zool.* **4**, 410 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Hollaender, Alexander	New developments in radiation biology	Institute of Biophysics, Rio de Janeiro
Karasaki, Shuichi	Electron microscope studies on the formation of nucleolus during amphibian embryogenesis [*J. Cell Biol.* **23**, 48A (1964)]	Am. Soc. Cell Biol., Cleveland
Kenney, F. T. (D. L. Greenman, W. D. Wicks, and W. L. Albritton)	RNA synthesis and enzyme induction by hydrocortisone	Third International Symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Tissues, Indianapolis, Ind.
Kimball, R. F.	Relation of the studies on mutagenesis in Paramecium to the dose rate problem	Conference on Mechanisms of the Dose Rate Effect at the Genetic and Cellular Level, Oiso, Japan
	Studies on mutation induction in Paramecium	1. Institute for Physical and Chemical Research, Tokyo 2. National Institute for Radiological Sciences, Chiba, Japan 3. Kyoto and Osaka Associations of Geneticists—Kyoto Assoc. of Radiation Biologists, Kyoto, Japan 4. Tohoku University, Sendai, Japan

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Kimball, R. F. (and Stella W. Perdue)	Use of conserved label in quantitative autoradiographic studies in *Paramecium aurelia* [*Am. Zool.* **4**, 385 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Kretchmar, A. L. (and C. C Congdon)	The metabolic problem in secondary disease	Colloque du Centre National de la Recherche Scientifique on Transplantation of Allogeneic Hemopoietic Cells, Paris
Leibo, S. P. (and Peter Mazur)	Relationship between freezing and osmotic shock of bacteriophage T4B	Biophysical Society, 9th Annual Meeting, San Francisco
Lindsley, D. L., Jr.	Chromosome function at the supragenic level	Symposium on Genes and Chromosomes, Buenos Aires
Malling, H. V.	The mutagenicity of the antineoplastic alkylating compounds	Department of Cancer Research, Municipal Hospital, Aarhus, Denmark
Manney, T. R.	Super-suppressible tryptophan synthetase mutants in yeast	University of Delaware, Newark
Mattingly, Mary E. (and K. M. Jakob)	Studies on mechanisms involved in 5-amino uracil-induced synchrony in *Vicia faba* [*J. Cell Biol.* **23**, 57A (1964)]	Am. Soc. Cell Biol., Cleveland
Mazur, Peter	Causes of injury in frozen and thawed cells	Am. Cancer Soc. Conference on Cryobiology, Rye, N. Y.
	Physical and chemical factors in the freezing injury of cells	Michigan State University, East Lansing
	The role of cell membranes in the freezing of single cells	New York Academy of Science Conference on Forms of Water in Biologic Systems, New York
	The role of water in the freezing of living cells	Oak Ridge National Laboratory, Oak Ridge, Tenn.
McGrath, R. A.	Radiation-induced incorporation of H^3TdR into DNA of *Physarum polycephalum* [*J. Cell Biol.* **23**, 58A (1964)]	Am. Soc. Cell Biol., Cleveland
McGrath, R. A. (and R. W. Williams)	Degradation of x-ray damaged DNA — a model for repair	Biophysical Society, 9th Annual Meeting, San Francisco
Miller, O. L., Jr.	Extrachromosomal nucleolar DNA in amphibian oocytes [*J. Cell Biol.* **23**, 60A (1964)]	Am. Soc. Cell Biol., Cleveland
	Fine structure of lampbrush chromosomes (and demonstration) [*J. Cell Biol.* **23**, 109A (1964)]	Am. Soc. Cell Biol., Cleveland
	Fine structure of lampbrush chromosomes	Symposium on Genes and Chromosomes, Buenos Aires
	Lampbrush chromosomes	Gordon Conference on Cancer, New London, N. H.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Muhammed, Amir	Purification of photoreactivating enzyme	Biophysical Society, 9th Annual Meeting, San Francisco
Novelli, G. D.	Current status of the problem of protein biosynthesis – I	1. Tulane University, New Orleans 2. University of Missouri, Columbia
	Current status of the problem of protein biosynthesis – II	Tulane University, New Orleans
	Protein synthesis in bacteria	Tulane University, New Orleans
	Protein synthesis by regenerating rat liver	1. Tulane University, New Orleans 2. University of Mississippi, Jackson 3. University of Tennessee, Memphis
Oakberg, E. F.	The effects of dose, dose rate, and radiation quality on the dynamics and survival of the spermatogonial population of the mouse	Conference on Mechanisms of the Dose Rate Effect at the Genetic and Cellular level, Oiso, Japan
	Radiation response of spermatogonia and oocytes	1. Dept. of Biology, Nuclear Research Center, Athens 2. Laboratorio di Radiobiologia animale, C.S.N. della Casaccia, Comitato Nazionale, Rome 3. Mount Vernon Hospital, Middlesex, England 4. Harwell, Didcot, Berkshire, England
Odell, T. T., Jr.	1. Acute radiation injury and recovery in mammals 2. Review of mammalian anatomy and physiology	ORNL-ORINS Ten-Week Course in Health Physics, Oak Ridge, Tenn.
Odell, T. T., Jr. (T. P. McDonald and C. W. Jackson)	Regulation of blood platelets	Xth Congress of the International Society of Haematology, Stockholm
Padilla, G. M.	Cell synchrony – its use in understanding the cell	University of Tennessee, Knoxville
	Effects of actinomycin D on synchronously dividing *Tetrahymena*	University of Tennessee, Knoxville
Padilla, G. M. (and G. L. Whitson)	Effects of actinomycin D on stomatogenesis and cell division in synchronized *Tetrahymena pyriformis*	Am. Soc. Zool., Knoxville, Tenn.
Padilla, G. M. (I. L. Cameron and O. L. Miller, Jr.)	Synchronization of cell division in *Tetrahymena pyriformis* by a repetitive temperature cycle	Cell Synchrony Conference, Oak Ridge, Tenn.
Randolph, M. L.	Comparison of kinetics of free radicals produced by neutron and x-ray irradiation of polycrystalline amino acids	Biophysical Society, 9th Annual Meeting, San Francisco
	Multi-section ESR cavities	Am. Physical Soc., SE Section, Chattanooga, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Regan, J. D.	Genetic studies of human amnion cells *in vitro*	Ohio University, Athens
Rogers, Stanfield	Concerning the mechanisms of action of carcinogens	University of Kentucky, Lexington
Russell, Liane B.	The mammalian X-chromosome	Washington University, St. Louis
Russell, W. L.	Genetic effects of radiation	1. Japan Radiation Research Society, Kyoto, Japan 2. The National Institute of Genetics, Misima, Japan 3. The Atomic Bomb Casualty Commission, Hiroshima, Japan
	The nature of the dose-rate effect of radiation on mutation in mice	Conference on Mechanisms of the Dose Rate Effect at the Genetic and Cellular Level, Oiso, Japan
Schnell, J. V.	Methods of cell breakage in relation to cellfree synthesis of peptide bonds	Stanford University, Palo Alto, Calif.
Schuel, Herbert (and N. G. Anderson)	Automated determination of protein in the presence of sucrose	Technicon International Symposium, New York
Setlow, Jane K. (F. J. Bollum and M. E. Boling)	Photoreactivable lesions in DNA	Biophysical Society, 9th Annual Meeting, San Francisco
Setlow, R. B.	Cytosine and thymine photoproducts in uv-irradiated DNA	Brookhaven National Laboratory, Upton, L. I., N.Y.
	Effects of uv on polynucleotides	University of Massachusetts, Amherst
	A molecular error-correcting mechanism	Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn.
	The ultraviolet irradiation of polynucleotides : physicochemical and biological effects [*Abstr. of Papers, 148th Meeting, American Chemical Society (1964)*, p. 21V]	Am. Chem. Soc., Chicago
	Ultraviolet irradiation of polynucleotides : physicochemical and biological effects	1. ORNL Laboratory Chemistry Colloquium, Oak Ridge, Tenn. 2. Union Carbide Research Laboratories, New York
Setlow, R. B. (W. L. Carrier and F. J. Bollum)	Dimers of cytosine and thymine residues in uv-irradiated polydeoxyribonucleotides	Biophysical Society, 9th Annual Meeting, San Francisco
Shepard, D. C.	Production and elimination of excess DNA in ultraviolet-irradiated *Tetrahymena* [*J. Cell Biol.* **23**, 86A (1964)]	Am. Soc. Cell Biol., Cleveland
Simmons, M. L.	Parasite screening and the therapeutic value of organic phosphates in inbred mice [*Lab. Animal Care* 14, 326 (1964)]	Animal Care Panel, 15th Annual Meeting, New York

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Smith, L. H.	Erythropoietic capacity of cultured bone marrow	Xth Congress of International Society of Haematology, Stockholm
Stapleton, G. E.	Lethal, mutagenic, and cytogenetic effects of fast charged particles on various biological materials	Second Symposium on Protection Against Radiations in Space, Gatlinburg, Tenn.
Stone, G. E. (and O. L. Miller, Jr.)	Incorporation of tritiated thymidine into deoxyribonuclease-sensitive material in mitochondria of *Tetrahymena* [*J. Cell Biol.* **23**, 89A (1964)]	Am. Soc. Cell Biol., Cleveland
Stone, G. E. (D. M. Prescott and O. L. Miller, Jr.)	Cytoplasmic DNA in *Tetrahymena* [*J. Protozool.* **11** (suppl.), 24 (1964)]	AIBS (Soc. Protozool.), Boulder, Colo.
Swartzendruber, D. C.	Electron microscopic observations on cellular changes in mouse spleen associated with an immune response	1. Louisiana State University, Baton Rouge, La. 2. Sam Houston State College, Huntsville, Tex. 3. University of Massachusetts, Amherst
	Electron microscopic studies on lymphatic tissue	Knoxville College, Knoxville, Tenn.
Swartzendruber, D. C. (and M. G. Hanna, Jr.)	Electron microscopic autoradiographic study of mouse spleen germinal centers [*Am. Zool.* **4**, 398 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Swenson, P. A. (and R. B. Setlow)	Inactivation of messenger RNA by ultraviolet irradiation	Society of General Physiologists, 19th Annual Meeting, Woods Hole, Mass.
Tipton, S. R. (Herbert Schuel and N. G. Anderson)	Automated analysis of cytochrome oxidase activity in rat liver brei	Technicon International Symposium, New York
Trosko, J. E.	Advances in cell biology	North Dakota State University, Fargo
	Studies on isolated chromosomes	Metabolism and Radiation Research Laboratory, Fargo, N. D.
Trosko, J. E. (and Sheldon Wolff)	Strandedness of mitotic chromosomes of *Vicia faba* as revealed by enzyme digestion studies [*J. Cell Biol.* **23**, 98A (1964)]	Am. Soc. Cell Biol., Cleveland
Trosko, J. E. (E. H. Y. Chu and W. L. Carrier)	In vivo induction of thymidine dimers in mammalian DNA [*Am. Zool.* **4**, 384 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Turney, T. H., Jr. (and K. B. Jacobson)	Lactic dehydrogenases of different molecular weight	Society of General Physiologists, 19th Annual Meeting, Woods Hole, Mass.
Upton, A. C.	Current trends in cancer research	6th IBM Medical Symposium, Poughkeepsie, N. Y.
	Recent developments in radiation carcinogenesis: mechanisms and implications	Health Physics Seminar, ORNL, Oak Ridge, Tenn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Van Dreal, Paul (and N. G. Anderson)	Ribosomes in *Astasia longa* and cell cycle variation in the T.C.A.-soluble nucleotide pool	Cell Synchrony Conference, Oak Ridge, Tenn.
von Borstel, R. C.	Spontaneous and induced mutations during mitosis and meiosis in yeast	Brooklyn College, New York
Walburg, H. E., Jr. (Edna I. Mynatt, G. E. Cosgrove, R. L. Tyndall, and D. M. Robie)	Studies on the microbiological effectiveness of a laboratory animal isolation facility [*Lab. Animal Care* **14**, 322 (1964)]	Animal Care Panel, 15th Annual Meeting, New York
Wald, Niel (S. Pan, A. C. Upton, V. K. Jenkins, and E. B. Darden)	Consistent chromosome aberrations in radiation-induced mouse granulocytic leukemia	Xth Congress of International Society of Haematology, Stockholm
Wallace, Robin	The formation and structure of vertebrate yolk granules	University of Massachusetts, Amherst
Warner, A. H.	Nucleotide metabolism during brine shrimp embryogenesis	Smith Kline and French Laboratories, Philadelphia
Warner, A. H. (and F. J. Finamore)	Hydrolysis of P^1P^4-diguanosine 5′-tetraphosphate by a new enzyme [*Abstr. of Papers, 148th Meeting, American Chemical Society (1964)*, p. 48C]	Am. Chem. Soc., 148th Annual Meeting, Chicago
Whiting, P. W.	A cytoplasmic factor in *Mormoniella* [*Am. Zool.* **4**, 405 (1964)]	Am. Soc. Zool., Knoxville, Tenn.
Whitson, G. L.	Morphogenetic studies on synchronized and regenerating cells	University of North Carolina, Chapel Hill, N. C.
Whitson, G. L. (G. M. Padilla and L. H. Elrod)	A long-lived RNA in synchronized *Tetrahymena pyriformis*	Biophysical Society, 9th Annual Meeting, San Francisco
	Morphogenesis, RNA synthesis, and actinomycin D inhibition in synchronized *Tetrahymena pyriformis* [*J. Cell Biol.* **23**, 102A (1964)]	Am. Soc. Cell Biol., Cleveland
Whitson, G. L. (G. M. Padilla and W. D. Fisher)	Actinomycin D inhibition in synchronized *Tetrahymena pyriformis*	Cell Synchrony Conference, Oak Ridge, Tenn.
Willard, Henrianne G.	Warm temperature preservation of mouse hemopoietic tissue	Society for Cryobiology, Washington, D. C.
Williams, G. R.	Control of RNA and protein synthesis by light	University of Georgia, Athens

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Wolff, Sheldon	Are sister chromatid exchanges sister strand crossovers or radiation-induced exchanges?	1. Brookhaven National Laboratory, Upton, L.I., N.Y. 2. Hebrew University, Jerusalem, Israel 3. University of Rome, Rome
	Mechanisms of dose rate effects: insights obtained from intensity and fractionation studies on chromosome aberration induction	Conference on Mechanisms of the Dose Rate Effect at the Genetic and Cellular Level, Oiso, Japan
	On the chemistry of chromosome continuity	1. "Democritus" Nuclear Research Center, Athens 2. Hebrew University, Jerusalem, Israel 3. Indian Agricultural Research Institute, New Delhi, India 4. International Laboratory of Genetics and Biophysics, Naples, Italy 5. National Institute of Genetics, Misima, Japan 6. Weizmann Institute, Rehovoth, Israel 7. Symposium on Genes and Chromosomes, Buenos Aires
	Studies on radiation genetics and radiation-induced chromosome aberrations (three lectures)	Stanford University, Palo Alto, Calif.
Wust, C. J.	The effect of actinomycin D on the immune response	Donner Laboratory, Berkeley, Calif.
	Studies on antibody biosynthesis	Brown University, Providence, R. I.
Wust, C. J. (and M. G. Hanna, Jr.)	The effect of actinomycin D on the immune response to two antigens given in sequence	Reticuloendothelial Society, New York
Yamada, Tuneo	An approach to cellular differentiation	University of Tennessee, Knoxville
	Immunological and autoradiographic studies of lens regeneration	Johns Hopkins University, Baltimore, Md.
	A study of cellular differentiation in lens regeneration	University of Virginia, Charlottesville
Yamada, Tuneo (and Chinami Takata)	The pattern of RNA synthesis as related to lens antigen formation during lens regeneration in the newt [*J. Cell Biol.* **23**, 104A (1964)]	Am. Soc. Cell Biol., Cleveland
Yamada, Tuneo (Chinami Takata and J. F. Albright)	Acquisition of macromolecular specificity in lens regeneration	International Conference on Organogenesis, Baltimore, Md.
Zimmerman, B. K.	A deoxyribonucleotide requiring exonuclease associated with the DNA polymerase from *Micrococcus lysodeikticus*	Biophysical Society, 9th Annual Meeting, San Francisco

VISITING LECTURERS

From August 16, 1964, to February 15, 1965, 48 lectures were given on the Biology Division Seminar Program by guest speakers from scientific institutions in this country and abroad. Lectures were given by scientists from Chile, England, France, Germany, Japan, the Netherlands, Sweden, and Switzerland.

SPEAKER	AFFILIATION	SUBJECT
B. C. Abbott	University of Illinois, Urbana	Excitation-contraction coupling in muscle
Richard D. Allen	Harvard University, Cambridge, Mass.	Fine structure of *Psilotum nudum* during cell division
Gunter F. Bahr	Department of Pathology, Armed Forces Institute of Pathology, Washington, D.C.	Quantitative cytochemistry with the electron microscope
Per-Erik Bergner	Karolinska Institute, Stockholm, Sweden	Interpretation of red cell survival data
W. Bernhard	Institut de Recherches Scientifiques sur le Cancer, Centre National de la Recherche Scientifique, Villejuif, France	Some recent problems of tumor virus research as seen by an electron microscopist
Alan K. Bruce	State University of New York at Buffalo	Sensitization of a radioresistant bacteria
A. J. Clark	Department of Bacteriology, University of California, Berkeley	The production, characterization and exploitation of bacterial mutants unable to catalyze genetic recombination
Robert Cumming	Department of Zoology, University of Texas, Austin	Dragonfly chromosomes
James N. Dumont	Department of Zoology, University of Massachusetts, Amherst	Histologic and cytologic changes in the crop of pigeons during crop-"milk" formation
Goro Eguchi	Department of Biology, Nagoya University, Nagoya, Japan	An electron microscope observation in Wolffian lens regeneration
Enrique Espinosa	Universidad de Chile, Escuela de Medicina, Catedra de Fisiopatologie, Santiago, Chile	Sex-associated differences in serum and liver proteins of mice
Setsuya Fujita	Purdue University, Lafayette, Ind.	Cellular proliferation and differentiation in the central nervous system
J. Gani	Department of Statistics, Michigan State University, East Lansing	Some stochastic models for phage reproduction
Ernst Hadorn	University of Zurich, Zurich, Switzerland	Manifold changes in the direction and capacity of differentiation occurring in permanent cultures of *Drosophila* cells
Yujiro Hayashi	Department of Biology, Nagoya University, Nagoya, Japan	Epithelio-mesenchymal interaction in the differentiation of skin derivatives
Raymond N. Hiramoto	St. Jude Hospital, Memphis, Tenn.	Detection of double antibody-producing cells by the paired fluorescence technique
Y. Hiramoto	University of Tokyo, Tokyo, Japan	Microsurgical work on fertilization and cleavage in sea urchin eggs

SPEAKER	AFFILIATION	SUBJECT
Virginia Holten	University of North Dakota, Grand Forks	Kinetic studies of lactate dehydrogenase
Elizabeth W. Jones	Department of Genetics, University of Washington, Seattle	Relative sizes of two adenine genes in yeast
Tsutomu Kasuga	Department of Pathology, University of Southern California, Los Angeles	Studies on melanogenesis
Paul Koch	University of Marburg, Marburg, Germany	Changing protein patterns in late developmental states of the stick insect egg in correlation to the growth of the embryo
Paul Kotin	National Cancer Institute, Bethesda, Md.	Studies in chemical carcinogenesis
Leon Krausz	Department of Chemistry, Princeton University, Princeton, N.J.	Hydrophobic bonding – models and methods
Albert I. Lansing	Department of Anatomy and Cell Biology, University of Pittsburgh, Pittsburgh, Pa.	Cell membranes in aging of rotifers
Robert Leif	Centre de Physique et de Chimie Colloidales, Montpellier, France	The buoyant density distribution of erythrocytes
John Lett	Chester Beatty Research Institute, London, England	Sensitization to x rays by BUdR in mammalian cells
Hans Lettre	University of Heidelberg, Heidelberg, Germany	Effects of some purine derivatives on normal and tumor cells
Renate Lettre	University of Heidelberg, Heidelberg, Germany	Morphological studies on the nucleolus of cells *in vitro*
A. Lima-de-Faria	Department of Zoology, Duke University, Durham, N.C.	Patterns of DNA replication in chromosomes of chronic granulocytic leukemia
John McLeish	Department of Cell Biology, John Innes Institute, Hertshire, England	Quantitative relationships between DNA and RNA in nuclei and nucleoli isolated from the roots of higher plants
Howard C. Mel	Donner Laboratory, University of California, Berkeley	Biophysical studies of hemopoietic and other cells by stable-flow free boundary migration
W. E. C. Moore	Virginia Polytechnic Institute, Blacksburg	Some newly discovered intestinal anerobic bacteria
Peter C. Nowell	Department of Pathology, University of Pennsylvania Medical School, Philadelphia	The long-lived lymphocyte
T. Okada	Carnegie Institute of Washington, Baltimore, Md.	Nephric tubulogenesis: an immunological study
P. G. Pershan	Harvard University, Cambridge, Mass.	Excited and metastable states in irradiated polynucleotides
Ch. Pilgrim	University of Köln, Köln, Germany	Studies of cell proliferation and DNA synthesis using autoradiography
Otto J. Plescia	Institute of Microbiology, Rutgers – The State University, New Brunswick, N.J.	Nucleic acids as antigens
M. Mark Ptashne	Biological Laboratories, Harvard University, Cambridge, Mass.	Lambda prophage and DNA transfer in bacterial mating

SPEAKER	AFFILIATION	SUBJECT
Ronald Rahn	Bell Telephone Laboratories, Murray Hill, N.J.	Singlet and triplet states in synthetic polynucleotides
Dan Ray	Stanford University, Stanford, Calif.	Properties of the satellite DNA's of *Euglena gracilis*
Amiram Ronen	University of California, San Diego	DES-induced mutation in *Salmonella typhimurium*
H. A. L. Trampusch	Anatomic-Embryological Institute, Amsterdam, The Netherlands	The effect of irradiation on embryonic properties
Paul O. Vogelhut	University of California, Berkeley	Structure of bound water in biological systems
Duard Walker	Department of Microbiology, University of Wisconsin School of Medicine, Madison	Persistent viral infection
Masaoki Yamada	Departments of Anatomy and Histology, Nara Medical College, Nara, Japan	A non-aqueous isolation of the cell nuclei and its application for microspectrophotometry and interferometry
H. G. Zachau	Institute of Genetics, University of Köln, Köln, Germany	Recent experiments with sRNA
R. S. d. Zaldivar	Department of Radiation Biology, University of Rochester, Rochester, N.Y.	Histopathogenesis and induction mechanisms of stomach neoplasia
Anders Zetterburg	Institute for Cell Research, Karolinska Institut, Stockholm, Sweden	Cytochemical studies on nucleic acid and protein synthesis during interphase in mouse fibroblasts *in vitro*

SPEAKERS AT PROFESSIONAL MEETINGS, SPRING–SUMMER 1965

Ninth Annual Meeting of the Biophysical Society – N. G. Anderson (coauthor, A. A. Barber), F. J. Bollum, C. L. Burger (coauthor, N. G. Anderson), I. L. Cameron (coauthors, G. M. Padilla and Betty M. Wysinger), L. G. Caro, J. E. Donnellan, Jr. (coauthor, R. B. Setlow), S. P. Leibo (coauthor, Peter Mazur), R. A. McGrath (coauthor, R. W. Williams), Amir Muhammed, M. L. Randolph, Jane K. Setlow (coauthors, F. J. Bollum and M. E. Boling), R. B. Setlow (coauthors, W. L. Carrier and F. J. Bollum), G. L. Whitson (coauthors, G. M. Padilla and L. H. Elrod), and B. K. Zimmerman

International Atomic Energy Agency Symposium on Personnel Dosimetry for Accidental High-Level Exposure to External and Internal Radiation – P. Carolyn Gooch

Japanese-American Conference in Developmental Biology – Tuneo Yamada

18th Annual Biology Research Conference – F. T. Kenney

56th Annual Meeting of the American Association for Cancer Research – V. K. Jenkins (coauthors, A. C. Upton and T. T. Odell, Jr.)

Histochemistry Society – R. C. Allen (coauthor, Dorothy J. Moore)

Federation of American Societies for Experimental Biology – Michael Bennett (coauthor, G. M. Shearer), F. J. Bollum, C. C Congdon, Gustavo Cudkowicz, J. L. Epler (coauthors, W. E. Barnett and K. B. Jacobson), Joan Wright Goodman, J. G. Green (coauthor, N. G. Anderson), A. D. Kelmers (coauthor, M. P. Stulberg), C. G. Mead (coauthor, Elliot Volkin), Paul Nettesheim (coauthor, Takashi Makinodan), G. D. Novelli (coauthors, Piero Cammarano, Marialuisa Melli, and G. Giudice), E. H. Perkins (coauthor, Toshiteru Morita), Stanfield Rogers, G. M. Shearer, Mayo Uziel (coauthor, W. E. Cohn), A. H. Warner (coauthor, F. J. Finamore), W. D. Wicks (coauthor, F. T. Kenney), Henrianne G. Willard (coauthor, L. H. Smith), and C. J. Wust (coauthors, J. B. Murphy and S. F. Carson)

CIBA Symposium on Preimplantation Stages of Pregnancy – Liane B. Russell

The Association of Southeastern Biologists – P. W. Whiting

American Association of Anatomists – D. C. Swartzendruber

American Society for Microbiology – H. I. Adler (coauthor, Alice A. Hardigree), Roy Curtiss III (coauthors, Claire M. Berg and L. J. Charamella), M. I. Dolin (coauthor, R. H. Baum), Alfred Hellman (coauthor, D. J. Giron), G. E. Stapleton (coauthor, W. D. Fisher), R. L. Tyndall (coauthors, K. B. Jacobson and Ernestine Teeter)

International Symposium on Immunological Tolerance – Takashi Makinodan

Radiation Research Society – J. G. Brewen, J. A. Heddle, R. F. Kimball, and Sheldon Wolff

Foreign Travel

Members of the Biology Division traveled to Asia, Europe, and South America during this report period (August 16, 1964–February 15, 1965). They attended and participated in meetings and conferences, presented lectures and seminars, and consulted with other scientists.

The Xth Congress of the International Society of Haematology was held in Stockholm, Sweden, August 30–September 4. As was the case in 1962, the sessions of the last day of this meeting were held in common with the first day's sessions of the Xth Congress of the International Society of Blood Transfusion. Five members of the Biology Division traveled to Stockholm to attend the meetings and present papers. At the Haematology meetings the following papers were presented:

C. C Congdon, M. A. Kastenbaum, and D. A. Gardiner	The extreme variation in mortality from secondary disease in radiation chimeras
Gustavo Cudkowicz	Functional characterization of the mouse marrow lymphocyte
T. T. Odell, Jr.	Regulation of blood platelets
L. H. Smith	Erythropoietic capacity of cultured bone marrow

At the Blood Transfusion meetings (September 3–8), the Division's speakers and their papers were:

Gustavo Cudkowicz	Lack of expression of parental isoantigen(s) in F_1 hybrid mice
Joan Wright Goodman	Differences in the specific immunological nonresponsiveness between genetically tolerant mice and those made tolerant by inoculation at birth

A paper coauthored by A. C. Upton, V. K. Jenkins, and E. B. Darden, Jr., of this Division was presented at the Haematology meetings by the senior author, Niel Wald, of the University of Pittsburgh.

After the Stockholm meetings, Drs. Congdon, Cudkowicz, Goodman, and Odell went to Paris, France, where they attended the CNRS Discussions on the Transplantation of Allogeneic Hematopoietic Cells, September 7–9. Dr. Congdon served as chairman of one of the sessions and was coauthor of a paper presented by A. L. Kretchmar of the Oak Ridge Institute of Nuclear Studies entitled "The metabolic problem in secondary disease." Other papers were:

Gustavo Cudkowicz	Hybrid resistance to parental hemopoietic cell grafts: implications for bone marrow chimeras
Joan Wright Goodman	Concerning the capacity of lymphocytes to give rise to erythropoiesis

Prior to the Stockholm meetings, Dr. Smith visited fellow investigators in London, England, and Paris, France. He consulted with colleagues at the Medical Biological Laboratory of the National Defense Research Council at Rijswijk, the Netherlands, where he had spent the period September 1960–September 1961, and also with Otto Vos, who spent a year in the Biology Division from October 1958 to October 1959 as a visiting investigator from abroad.

Dr. Cudkowicz traveled to Italy and Israel after the Paris meeting. In Italy he visited the Universities of Milan and Pavia and at the latter institution presented a seminar entitled "Genetic interaction in the inheritance of mouse histocompatibility factors." In Israel, he again spoke on "Genetic interaction in the inheritance of mouse histocompatibility factors" at the Department of Cell Biology, Weizmann Institute of Science, Rehovoth, and on "Lack of expression of parental isoantigen(s) in F_1 hybrid mice" at the Hebrew University in Jerusalem.

In September, W. E. Cohn served as United States delegate at a joint meeting of the Commissions on the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB). The purpose of the meeting, held in Salzburg, Austria, was to formulate nomenclature in biochemistry. Dr. Cohn was elected secretary of the combined commission.

H. V. Malling and C. M. Steinberg spent the week of October 18–25 visiting colleagues abroad. Dr. Malling went to the Chester Beatty Research Institute and to University College in London, England, and also visited the Department of Cancer Research, Municipal Hospital, Aarhus, Denmark, where he lectured on "The mutagenicity of the antineoplastic alkylating compounds." Dr. Steinberg visited the Institut de Biologie Moleculaire, University of Geneva, Switzerland.

E. H. Y. Chu spent October 8–November 8 in Taiwan and Japan. During the first week, he visited the Academia Sinica, the National Taiwan University, and the National Defense Medical College. He lectured on the "Induction of mammalian chromosomes by ultraviolet light" at the Academia and conducted a joint seminar on "Mammalian chromosome cytology" for members of the Departments of Botany, Agronomy, and Medicine at the University. On October 15, Dr. Chu traveled to Japan, where he visited laboratories and discussed mammalian cytology and human cytogenetics with colleagues at the Zoological Institute, Hokkaido. While there, he again presented "Induction of mammalian chromosomes by ultraviolet light." He reviewed and advised on the human cytogenetics program at the Atomic Bomb Casualty Commission and visited the University of Hiroshima Medical School at Hiroshima; he discussed genetics at the University of Osaka and consulted on radiation effects on mammalian cells in vitro and microbial genetics at the University of Kyoto. He gave lectures at both universities. Next, Dr. Chu traveled to Tokyo, where he discussed a number of aspects of radiation genetics and human genetics with investigators at the National Institutes of Radiological Sciences at Chiba, at the National Institute of Health, at the University of Tokyo, and at the Tokyo Medical and Dental University.

On November 1, Dr. Chu proceeded to Misima, where he consulted with investigators at the National Institute of Genetics. From Misima he went to Oiso where he joined other members of the Biology

Division attending the Conference on Mechanisms of the Dose Rate Effect at the Genetic and Cellular Level, a meeting held under the joint sponsorship of the United States–Japan Cooperative Science Program (National Science Foundation) and the National Institute of Genetics, Misima, Japan. Division members attending the meeting and the titles of the invited papers given by each were:

R. F. Kimball	Relation of the studies on mutagenesis in Paramecium to the dose rate problem
E. F. Oakberg	The effects of dose, dose rate, and radiation quality on the dynamics and survival of the spermatogonial population of the mouse
W. L. Russell	The nature of the dose-rate effect of radiation on mutation in mice
Sheldon Wolff	Mechanisms of dose rate effects: insights obtained from intensity and fractionation studies on chromosome aberration induction

R. F. Kimball, who served as coordinator for the United States delegation to the Conference, went first to Tokyo, where he discussed the United States–Japan Cooperative Science Program. After the Oiso meeting, he visited the National Institute of Genetics, Misima; Tohoku University, Sendai; the Research Institute for Physics and Chemistry, Tokyo; the National Institute for Radiological Sciences, Chiba; and Kyoto University, Kyoto. At all these institutions Dr. Kimball consulted with research personnel and toured the laboratories. While in Kyoto, he spoke on "Studies on mutation induction in Paramecium" at a joint meeting of the Kyoto and Osaka Associations of Geneticists and the Kyoto Association of Radiation Biologists. This lecture was also presented at the Institute for Physical and Chemical Research, Tokyo; the National Institute for Radiological Sciences, Chiba; and Tohoku University, Sendai.

E. F. Oakberg spent the week prior to the Oiso meeting visiting laboratories in Tokyo, Chiba, and Misima. He discussed radiation biology and genetics, gametogenesis, dose rate and cell stage effects, and related matters with fellow investigators. After the meeting, he traveled to Hiroshima where he consulted with colleagues at the Atomic Bomb Casualty Commission. He concluded his stay in Japan with visits to the University of Osaka; Nara Medical College, Nara; and the University of Kyoto. From Japan, Dr. Oakberg went to Israel, and visited the Hebrew University in Jerusalem and Tel Aviv for further discussions with fellow scientists before leaving for Europe. In Europe, Dr. Oakberg discussed radiation biology with fellow investigators at the Department of Biology, Nuclear Research Center, Athens, Greece; DNA, RNA, and protein metabolism of spermatogonia with colleagues at the Laboratorio di Radiobiologia animale, in Rome, Italy; cell survival data at the Mount Vernon Hospital, Middlesex, England; and radiation genetics at the British Atomic Energy Laboratory, Harwell, Berkshire, England. At all four institutions Dr. Oakberg delivered a seminar entitled "Radiation response of spermatogonia and oocytes."

In addition to the paper he presented at the Oiso meeting, W. L. Russell served as chairman at a conference on cooperative research for Japanese and American scientists. He also gave invited lectures on "Genetic effects of radiation" at a meeting of the Japan Radiation Research Society in Kyoto, at the National Institute of Genetics, Misima, Japan, and to the Atomic Bomb Casualty Commission, Hiroshima, Japan. While in Japan, Dr. Russell visited laboratories in Tokyo, Chiba, Misima, Hiroshima, Osaka, Nara, and Kyoto, at each of which he conferred with investigators on recent advances in radiation genetics, genetic effects in populations, hazards of radiation, and related matters. Dr. Russell, who has been elected president of the Genetics Society of America for 1965, also conferred with officers of the Genetics Society of Japan regarding the next International Congress of Genetics, which is to be held in Japan.

During his stay in Japan, Sheldon Wolff visited the National Institute of Genetics, Misima, and the University of Kyoto. While in Misima, he presented a lecture, "On the chemistry of chromosome continuity." After the meeting at Oiso, Dr. Wolff traveled to New Delhi, India, where he presented the same lecture and conferred with investigators at the Division of Botany, Indian Agricultural Research Institute, on genetic effects induced by irradiated media and on the effects of both radiation and chemical mutagens on plant and mammalian chromosomes. From India, Dr. Wolff went to Israel. At Hebrew University, Jerusalem, he delivered two seminars, "Are sister chromatid exchanges sister strand crossovers or radiation-induced exchanges?" and "On the chemistry of chromosome continuity." He also discussed genetic and cellular effects of radiation with colleagues there. Then he visited the Weizmann Institute in Rehovoth, again presented "On the chemistry of chromosome continuity," and left for Europe.

In Europe, Dr. Wolff discussed chromosomal aberrations with scientists at the "Democritus" Nuclear Research Center in Athens, Greece, at the University of Rome, Rome, Italy, and at the International Laboratory of Genetics and Biophysics, Naples, Italy. In Greece, he lectured on "On the chemistry of chromosome continuity"; in Rome, on "Are sister chromatid exchanges sister strand crossovers or radiation-induced exchanges?"; and in Naples on "On the chemistry of chromosome continuity." He also visited the Italian Atomic Energy Commission at Casaccia.

In late November, Dr. Wolff left Europe for South America to attend the Symposium on Genes and Chromosomes – Structure and Function, which was held in Buenos Aires November 30–December 4 under the sponsorship of the Pan American Union. There he joined seven other Division members, including Alexander Hollaender, who served as president of the Symposium and presented the opening and closing remarks. Dr. Wolff and Dr. D. L. Lindsley were members of the organizing committee. Titles of papers presented by Division members were:

Sheldon Wolff	On the chemistry of chromosome continuity
F. J. de Serres	Impaired complementation between nonallelic mutations in Neurospora
Rhoda F. Grell	Chromosome pairing, crossing over, and segregation in *Drosophila melanogaster*
D. L. Lindsley	Chromosome function at the supragenic level
O. L. Miller, Jr.	Fine structure of lampbrush chromosomes

Two other Division members, M. A Bender and E. H. Grell, also attended the Symposium. Dr. Bender had traveled to South America earlier in November. After a brief visit with colleagues at the University of Brazil in Rio de Janeiro, he went to Buenos Aires to attend the Eighth International Congress of Internal Medicine, held November 23 through 28, and presented a paper entitled "Cytogenetic Effects of Radiation in Man." Then he attended the Symposium on Genes and Chromosomes – Structure and Function. Following the Symposium, he revisited the University of Brazil in late November and December.

After the Symposium on Genes and Chromosomes, other participants visited colleagues in various laboratories and institutions.

Dr. de Serres visited genetics laboratories in São Paulo, Rio de Janiero, and Brasilia for discussions on research problems.

Rhoda F. Grell and E. H. Grell remained in Buenos Aires for two and one-half months at the invitation of the Argentine Atomic Energy Commission and the University of Buenos Aires. They collaborated with Drs. Juan and Ruby Valencia.

D. L. Lindsley visited facilities at the University of São Paulo, the Institute of Biophysics in Rio de Janeiro, and the University of Brazil in Brasilia.

On December 5, Alexander Hollaender and O. L. Miller, Jr., went to Montevideo, Uruguay, to confer with Dr. Francisco A. Saez on plans for the Symposium on the Nucleolus, Its Structure and Function to be held in Montevideo December 6–10, 1965. Following this discussion, Dr. Hollaender visited the Institute of Biophysics of the University of Brazil, and gave an invited seminar on "New developments in radiation biology."

N. G. Anderson visited Sweden, Denmark, France, England, and Scotland for a series of discussions concerning zonal centrifugation. His trip began in late November and ended in late December 1964. Dr. Anderson was in Uppsala, Sweden, November 30 to December 1 for discussions with Arne Tiselius and Stig Claesson, Departments of Biochemistry and Physical Chemistry, Uppsala University; in Stockholm, Sweden, December 2–3, with T. Caspersson, Karolinska Institutet; in Copenhagen, Denmark, December 4–5 with Erik Zeuthen, Carlsberg Foundation; in Paris, France, December 6–7 with W. Bernhard, Institut de Recherches sur le Cancer; and in England and in Scotland December 7–20.

While in England and Scotland, Dr. Anderson visited Roy Markham on December 11 and 12 at the Plant Virus Laboratory at Cambridge University, England, and consulted with L. V. Crawford and Michael Stoker at the Department of Virology, University of Glasgow, Glasgow, Scotland, December 18 and 19. The remainder of the time was spent at the Chester Beatty Research Center with Peter Alexander.

During his trip, Dr. Anderson presented a lecture entitled "The development of zonal centrifuges" at Uppsala University, Sweden; the Karolinska Institutet, Stockholm; the Institut de Recherches sur le Cancer, Paris; the Carlsberg Foundation, Copenhagen; the Plant Virus Laboratory, Cambridge; the University of Glasgow, Glasgow; and the Ciba Foundation, London.

In February, J. L. Liverman was a member of a team from AEC's Division of Biology and Medicine making a site visit to Costa Rica.

As former chief of AEC's Biology Branch, he was the scientific representative with responsibility of program direction for the contract with Inter-American Institute of Agricultural Sciences at Turrialba. He was asked to make the visit because of his extensive knowledge of the program.

Meetings and Conferences

1965 BIOLOGY SYMPOSIUM

The eighteenth annual Research Conference sponsored by the Biology Division of the Oak Ridge National Laboratory will be held April 5–8 at the Riverside Motor Lodge, Gatlinburg, Tennessee. Attendance is by invitation only. The organizing committee, composed of F. T. Kenney, chairman, A. H. Haber, K. B. Jacobson, D. L. Lindsley, R. C. von Borstel, and Tuneo Yamada, has announced the following program:

HORMONAL CONTROL OF PROTEIN BIOSYNTHESIS

Monday, April 5

Control of Enzyme Levels

Morning

Opening Remarks – Alexander Hollaender

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

Introduction – Francis T. Kenney, Chairman, Organizing Committee

Chairman – W. Eugene Knox, Cancer Research Institute, New England Deaconess Hospital, Boston, Massachusetts

Nathan O. Kaplan and Theodore L. Goodfriend, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts. *Effect of Hormones on Lactic Dehydrogenases.*

Harold L. Segal and Yee S. Kim, Department of Biology, State University of New York, Buffalo. *Glucocorticoid Effect on the Synthesis of Glutamic-Alanine Transaminase in the Liver.*

Afternoon

Chairman – Sidney P. Colowick, Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee

Alberto Sols, José Salas, and Antonio Sillero, Department of Enzymology, Instituto Marañón, Centro de Investigaciones Biológicas, Madrid, Spain. *Insulin-Dependent Synthesis of Glucokinase.*

Henry Lardy, David Foster, Jerry Young, Earl Shrago, and Paul Ray, Institute for Enzyme Research, University of Wisconsin, Madison. *Hormonal Control of Enzymes Participating in Gluconeogenesis and Lipogenesis.*

J. E. Varner, Research Institute for Advanced Studies, Baltimore, Maryland. *Gibberellic Acid–Controlled Synthesis of α-Amylase in Barley Endosperm.*

Tuesday, April 6

Control of Chromosome Activity

Morning

Chairman – Crodowaldo Pavan, Department of General Biology, University of São Paulo, São Paulo, Brazil, and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Peter Karlson, Institute of Physiological Chemistry, University of Marburg, Marburg, Germany. *Biochemical Studies of Ecdysone Control of Chromosomal Activity.*

James Bonner, Division of Biology, California Institute of Technology, Pasadena. *Hormonal Effects upon the Template Activity of Chromatin.*

Afternoon

(Free)

Wednesday, April 7

Control of RNA Synthesis

Morning

Chairman – Elliot Volkin, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Jack Gorski, William D. Noteboom, and John A. Nicolette, Department of Physiology and Biophysics, University of Illinois, Urbana. *Estrogen Control of Uterine RNA and Protein Synthesis.*

H. G. Williams-Ashman, Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland. *Androgenic Control of RNA and Protein Synthesis in Male Accessory Genital Organs.*

Afternoon

Chairman – G. David Novelli, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Francis T. Kenney, Biology Division, Oak Ridge National Laboratory. *Hydrocortisone Stimulation of RNA Synthesis in Induction of Hepatic Enzymes.*

Gordon M. Tomkins, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland. *On the Regulation of Enzyme Synthesis by Steroid Hormones.*

A. Korner, Department of Biochemistry, University of Cambridge, Cambridge, England. *Growth Hormone Effects on RNA and Protein Synthesis in Liver.*

Thursday, April 8

Control in Neoplastic Tissues

Morning

Chairman – Philip P. Cohen, Department of Physiological Chemistry, University of Wisconsin Medical School, Madison

H. C. Pitot, Y. S. Cho, C. Lamar, and G. Moyer, McArdle Memorial Laboratory, University of Wisconsin Medical School, Madison. *Hormonal Control of Enzyme Levels in Rat Hepatomas.*

Van R. Potter, McArdle Memorial Laboratory, University of Wisconsin Medical School, Madison. *Conference Summary.*

General Discussion

Final Summation – Van R. Potter

ANNUAL INFORMATION MEETING

The Oak Ridge National Laboratory Advisory Committee for Biology made its annual visit to the Division November 18–20. The committee was composed of C. E. Carter, Western Reserve University Medical School; 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.

Division investigators summarized their work for the visitors at a conference in the Division on November 18 and 19. The committee members also visited individual laboratories and participated in informal discussions during their visit.

BIOMEDICAL DIRECTORS MEETINGS

The October 12–13 meeting of the Biomedical Program Directors of the Division of Biology and Medicine, United States Atomic Energy Commission, was attended by S. F. Carson and H. I. Adler at Hanford Laboratories, Richland, Washington.

The meeting held February 7–9, 1965, was attended by Alexander Hollaender in San Juan, Puerto Rico.

BONE MARROW CONFERENCES

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

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

Three conferences in this continuing series were held in 1964. The first 1964 conference, *Radiation Protection and Recovery Discussion Meeting,* was held April 13 in Chicago, under the chairmanship of the Division's R. A. Popp.

The other two bone marrow meetings held during this report period, include:

September 7–9, 1964, Paris, France – *The Transplantation of Allogeneic Hemopoietic Cells.* The Paris meeting was organized by Georges Mathé, Jean-Louis Amiel, and Léon Schwarzenberg, of the Institute of Cancerology and Immunogenetics, Hôpital Paul Brousse, Villejuif, Seine. Joan Wright Goodman and Gustavo Cudkowicz presented papers at this conference, while C. C Congdon, who coauthored a paper given by A. L. Kretchmar of the Oak Ridge Institute of Nuclear Studies, served as cochairman of the session on "Tolerance of the Host with Regard to the Graft." T. T. Odell, Jr., also attended.

December 4–5, 1964, Houston, Texas – *Radiation Protection and Recovery Conference.* The Houston conference was organized by Daniel E. Bergsagel, Daniel Billen, and Carl F. Tessmer of the University of Texas M. D. Anderson Hospital and Tumor Institute, and John Trentin, Baylor University College of Medicine. The Division's C. C Congdon and M. G. Hanna, Jr., presented papers at this conference. Coauthors of Dr. Congdon's paper are M. A. Kastenbaum and D. A. Gardiner. Carl J. Wust is coauthor of the paper presented by Dr. Hanna.

An evening session is being arranged for April 10, 1965, in Atlantic City, New Jersey, at the time of the annual meeting of the Federation of American Societies for Experimental Biology. Leonard J. Cole, United States Naval Radiological Defense Laboratory, will be chairman for this session.

Plans are also being formulated for a two-day conference to be held in November or December 1965 in San Francisco, California. Emphasis will be placed on cell separation studies as a special part of this conference.

In 1966, in addition to the spring and early winter meetings, a two-day discussion of the germinal centers of the lymphatic tissue is contemplated for June 22–23 (tentative dates) in Bern, Switzerland. This discussion meeting is scheduled to precede the Third International Congress of Radiation Research which meets in Cortina, Italy, starting June 26, 1966.

CELL SYNCHRONY CONFERENCE

Thirty cell biologists from the United States and abroad participated in the Division's Conference on Cell Synchrony October 9–10, 1964, at the Biology Division. The conference theme covered different methods of cell synchrony, and the physiological, biochemical, structural, and mathematical aspects of synchronization. The meeting was organized by G. M. Padilla (chairman), I. L. Cameron, and G. L. Whitson, and sponsored by the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee. Four papers were presented by Division members G. M. Padilla (coauthors I. L. Cameron and O. L. Miller, Jr.), I. L. Cameron, P. A. Van Dreal (coauthor N. G. Anderson), and G. L.Whitson (coauthors G. M. Padilla and W. D. Fisher). Abstracts of the papers have been published in a Biology Division booklet (Small Conference Booklet Number 11).

Educational Activities

Student Trainee Program. – Applications for the Summer Student Trainee Program have been reviewed, and 15–20 undergraduates will be selected to participate in 1965. In addition, a small group of students who have just received a bachelor's degree and have been accepted by a graduate school will be selected to work in the Division for the summer.

Small College Program. – The Small College Program, set up by the Biology Division, is basically an effort by the Division to arrange visits and lectures at small colleges. During this report period, two lectures were presented under this program. D. G. Doherty presented "Enzymes, the biological catalysts" at Winthrop College, Rock Hill, South Carolina, in December, and W. D. Gude presented "Biological effects of radiation" at Randolph-Macon Woman's College, Lynchburg, Virginia, in February.

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 Powell High School, Powell, Tennessee, December 1, and at Lenoir City High School, Lenoir City, Tennessee, January 19, 1965.

A three-day course, "Zonal Centrifugation Training Conference," was held at the Oak Ridge National Laboratory and the Oak Ridge Gaseous Diffusion Plant, September 16–18, 1964, to acquaint interested research personnel with the operation of the B-IV zonal ultracentrifuge system. The course was organized by N. G. Anderson, Biology Division, and coordinated through F. C. Von der Lage, Office of Industrial Cooperation. At the conference, N. G. Anderson presented a review of zonal centrifuge development and reported on other phases of zonal centrifugation research. Other Biology Division members who participated were W. D. Fisher, A. A. Barber, G. B. Cline, G. L. Whitson, and G. M. Padilla.

Sheldon Wolff presented three lectures as part of a course in radiobiology at Stanford University Medical School in October. The lectures were on "Studies on radiation genetics and radiation-induced chromosome aberrations."

In October, G. E. Cosgrove and T. T. Odell, Jr., participated in the ORINS-ORNL Training Division ten-week course in Health Physics. Dr. Cosgrove lectured on "Delayed somatic effects in mammals." Dr. Odell spoke on "Review of mammalian anatomy and physiology" and on "Acute radiation injury and recovery in mammals."

In October, G. D. Novelli presented a series of four lectures for graduate students at Tulane University, New Orleans, Louisiana. The lectures were entitled:

1. Protein synthesis in bacteria
2. Protein synthesis in regenerating rat liver
3. Current status of the problem of protein biosynthesis – I
4. Current status of the problem of protein biosynthesis – II

Two Division members lectured in the 15th Annual Navy Nuclear Sciences Seminar in December. The Seminar was sponsored by the Office of Naval Research, Department of the Navy, and was conducted by the local Naval Research Reserve Unit in conjunction with the Atomic Energy Commission's Oak Ridge Operations, the Oak Ridge National Laboratory, and the Oak Ridge Institute of Nuclear Studies.

Biology Division speakers and their subjects were: G. E. Cosgrove, "Radiation hazards to man"; and N. G. Anderson, "The zonal centrifuge."

Ernestine Teeter attended a training course January 26 to February 5, 1965, entitled "Serology of Indigenous Murine Viruses." The course was offered at the Communicable Disease Center, Atlanta, Georgia.

A. H. Haber presented "The role of cell division in morphogenesis" on January 5 as part of a "tele-lecture" series on meristems and differentiation in the Department of Biological Sciences at Purdue University. Dr. Haber gave his lecture by telephone from his Biology Division office. His voice was relayed to his Purdue audience of graduate students and other interested persons by a loud speaker arrangement. Slides and abstracts had been sent to the University and were used during his presentation.

University Cooperative Program. – Two Biology Division members are associated with the University of Tennessee in a part-time teaching and advisory capacity under the joint UT–Oak Ridge National Laboratory Cooperative Research Program. H. I. Adler is teaching bacterial genetics and making selected lectures in introductory bacteriology and is advising graduate students in research problems. He is associated with the University's Department of Bacteriology. A. H. Haber has been appointed, effective March 1, to a similar position in the University's Department of Botany. He will teach and advise students on subjects in the area of developmental plant physiology.

Introductory Course in Modern Zoology. – Several Biology Division members have contributed to the formation of a newly organized "Introductory Course in Modern Zoology," now being taught at the Oak Ridge Institute of Nuclear Studies in cooperation with the Biology Division and the University of Tennessee.

An increasing interest in cooperative programs concerning biomedical research within the total Oak Ridge research effort was a major reason for organizing the course. Many ORNL and AEC staff members – administrators, scientists, and technicians primarily from nonbiological fields – had expressed interest in taking courses in biology but lacked necessary prerequisites. As one step toward meeting this need, the new zoology course was designed specifically to train Oak Ridge nonbiological personnel in basic biology.

In general, the course schedule is planned for college graduates in nonbiological fields and with no previous courses in zoology. Subject matter includes the following: molecular biology, intermediary metabolism, cell substructure, organization of multicellular systems, cellular and organismal control systems, zoological taxonomy, structure and function of animal systems, morphological and chemical embryology, and principles of genetics and evolution.

A second purpose of the course is to prepare students interested in taking upper division university courses in biology. Persons who complete the course may take 300-level courses in biology and biochemistry (assuming chemistry requirements are met) at the University of Tennessee in future years.

The new course was organized by the Division's N. G. Anderson and G. L. Whitson, and has been directed by Dr. Whitson. The subject matter is covered in three ten-week quarters which correspond to the University's quarter system. College credit of 4 hr per quarter may be earned. Meetings are held at night for the convenience of professional participants.

Biology Division members who have lectured and conducted lab sessions thus far are N. G. Anderson, W. E. Barnett, I. L. Cameron, D. G. Doherty, L. H. Elrod, W. D. Fisher, W. D. Gude, G. M. Padilla, R. A. Popp, and G. L. Whitson. Other Division members, including Alexander Hollaender, Roy Curtiss III, F. J. Finamore, T. R. Manney, and Peter Mazur, have agreed to participate in later quarters. Tentative plans call for the series to be offered again in 1965–1966.

1. Cytology and Genetics

R. F. Kimball

Effects of Radiation on Paramecium (1.1–1.5)

R. F. Kimball[a]
Satomi Igarashi[b]
G. L. Whitson[c]
E. G. Bailiff
Barbara R. Bussell[a]
Stella W. Perdue
Carrie L. Wells

Phage Genetics (1.6–1.10)

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

Chromosome Cytology (1.11–1.15)

Sheldon Wolff
M. Ellen Mattingly
Sandra L. Bell[e]
J. A. Heddle[b,f]
K. M. Jakob[b]
J. E. Trosko[g]
H. E. Luippold
Agnes R. Patterson

Fungal Genetics (1.16–1.20)

F. J. de Serres[a]
H. V. Malling[a]
B. B. Webber[a]
B. J. Kilbey[a,b]
D. S. Carroll[a]
Mary A. Conner[a]
Anita M. Meley[a]
Ida C. Miller
C. Jean Painter
Marilyn T. Sheppard
Arlee P. Teasley[a]

Cooperative NIH-AEC Co-Carcinogenesis Program (1.18, 1.20)

Mammalian Genetic Cytology (1.21–1.23)

E. H. Y. Chu[a]
J. D. Regan[c]
Lana T. Covington
Margaret K. Kasschau[a]
Nina S. Hammer[a]

Mammalian Biochemical Genetics (1.24–1.26)

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

[a]Dual assignments.
[b]Visiting investigator from abroad.
[c]USPHS Fellow.
[d]Leave of absence, Stanford University.
[e]Consultant.
[f]James Picker Foundation Fellow.
[g]American Cancer Society Fellow.

1.1 DISTRIBUTION OF TEM-INDUCED MUTATIONS TO CHROMOSOME STRANDS[1]

R. F. Kimball Barbara R. Bussell
E. G. Bailiff

In the previous report[2] evidence was summarized to show that the alkylating agent, triethylenemelamine (TEM), produces mutations by way of reparable premutational damage. This damage is apparently subject to repair until the first post-treatment DNA synthesis, but not thereafter. In this, and in several other respects, it resembles the premutational damage produced by x rays as studied in our earlier work. The conclusion was drawn that the cell has the ability to repair a variety of different initial premutational lesions. This conclusion was based upon the assumption that the initial damage produced by x rays is different from that produced by TEM. Although this assumption seemed very probable, a priori, direct proof for it was needed.

One of the properties of mutations that should depend on the nature of the initial damage is the distribution of the mutation to the chromosome strands. X-ray-induced mutations in *Paramecium* and elsewhere normally affect all four conserved strands (the two old strands and the two newly synthesized ones) that are present at some stage of the irradiated cell generation.[3] Chemical mutagens have been reported to affect only part of the strands. If such a difference existed between the mutations induced by x rays and TEM, it would be strong evidence that the initial premutational damage was different.

The distribution of mutations to the strands was inferred, as in our previous work,[3] from the distribution to the progeny of the first few cell divisions. During these divisions, the strands are believed to segregate from one another in such a way that each of the four daughter products of the first two posttreatment divisions gets one and only one of the four conserved strands present during the treated cell generation. Therefore, the procedure was to separate the daughter products of each of several successive divisions into separate containers and test each product for the presence of a mutation. With this procedure, about two-thirds of all the mutations induced by x rays were found in all daughter products of those specimens that had any mutations at all (whole mutations). The other third of the mutations was found in all progeny from one daughter product of the first division and not in the other daughter product (half mutations). Thus, x rays affect either all conserved strands or, less frequently, one old and its corresponding new strand. Mutations affecting a smaller fraction of the progeny were no more frequent than in the controls.

One experiment with mutations induced by TEM yielded two whole, nine half, and eight quarter mutations, with one whole and four quarters in the untreated controls. A second experiment, in which only one product of the first division was saved and so the distinction between half and whole mutations could not be made, yielded six whole or half mutations, two quarters, and four eighths, with one whole or half and one eighth in the controls.

Despite the small numbers, the conclusion is clear. Most of the mutations induced by TEM are fractionals, including not only halves but smaller fractionals as well. Thus the initial damage produced by TEM must differ from that produced by x rays, and our earlier conclusion that the cell has the ability to repair a variety of different initial premutational lesions is given added support.

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1.2 ATTEMPTS TO INDUCE MUTATIONS IN *PARAMECIUM* WITH 3,4-BENZPYRENE[1]

R. F. Kimball E. G. Bailiff
Barbara R. Bussell

The idea that mutations are somehow responsible for carcinogenesis has been frequently expressed. Yet, some of the most active carcinogens, notably the carcinogenic hydrocarbons, have not been shown to be mutagens, and a number of mutagens have not been shown to be carcinogens.[2] Unfortunately, the tests for carcinogenicity and mutagenicity must be performed upon different cells and tissues, since no materials really suitable for both purposes have yet been found. Consequently, it is conceivable that negative results in one of the two kinds of activity may reflect some difference in response of the particular cells used rather than a fundamental inability of the agent to act in both ways. For this reason it seems desirable to test for mutagenic action of carcinogens in as wide a variety of objects as possible. It would be of some additional value if the object in question was also suitable for tests of other actions of the carcinogen. Such an object is *Paramecium aurelia*, in which studies of mutation can readily be combined with studies of the cell cycle and of intraclonal inheritance.

Therefore we have performed a series of tests for the induction of recessive lethal and slow-growth mutations by the potent carcinogenic hydrocarbon 3,4-benzpyrene. Through the courtesy of H. V. Malling of the *Neurospora* Genetics Group, solutions of 1 mg/ml of benzpyrene in 10% bovine

serum albumin and 10% TEG were obtained and diluted to one sixty-fourth of their initial concentration with culture medium (lettuce infusion with *Aerobacter aerogenes*) to make it possible for the paramecia to grow in the presence of the mutagen. Such solutions had only a small effect on the growth of paramecia kept in them for as long as one day. Illumination with long-wavelength ultraviolet light (above 300 mμ) produced a marked delay in division, a photodynamic effect of the hydrocarbon.[3]

Attempts to produce mutations either by a rellatively brief exposure (1 hr or less) to the hydrocarbon at various stages in the cell cycle or by overnight exposure through several cell cycles were unsuccessful. Attempts to produce mutation by the photodynamic action were also unsuccessful, whether the paramecia had been exposed to the hydrocarbon only briefly or overnight before exposure to the ultraviolet light.

Examination of the living paramecia with the fluorescence microscope showed a possible reason for this failure. The typical blue fluorescence of 3,4-benzpyrene in the presence of protein was readily visible in the cytoplasm but not in the nucleus. The nuclear membrane in this species is supposed to remain intact even through mitosis, in contrast to its breakdown in higher organisms. Thus the fact that it acts as a barrier to benzpyrene could account for the negative results. There is some, though not very adequate, evidence that the membrane may partially break down during meiosis. Thus, treatment during meiosis may be a better way to test for mutagenicity, and a preliminary experiment has been performed to discover some of the technical problems in this procedure.

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1.3 MICRONUCLEAR RNA SYNTHESIS AND MUTATION INDUCTION

R. F. Kimball Stella W. Perdue

In *Paramecium aurelia*, very few mutations can be induced when the micronucleus (the nucleus in which mutations can be detected) is in postduplication interphase (G_2) or early prophase.[1] It seemed likely that this refractoriness to mutation induction is somehow related to the DNA synthetic cycle, but other activities of the micronucleus also needed to be examined, and we have now completed a study of micronuclear RNA synthesis during the cycle.

Paramecia were treated in various known stages of the cell cycle by picking out groups of dividing cells from log-phase cultures and allowing different times to elapse between division and treatment. Part of each group was x irradiated and eventually scored for mutation yield. The other part was put for 15 min into a solution containing 100 μc/ml each of ^{3}H-cytidine and ^{3}H-uridine. We have already shown that neither ribonucleoside is incorporated into DNA under our conditions. At the end of the 15-min interval, the paramecia were fixed with 3% glutaraldehyde in 0.05 *M* phosphate buffer at pH 7.2, embedded in glycol methacrylate, and sectioned at 0.5 μ with a Porter-Blum microtome.

Autoradiographs were prepared, and grain counts were made over the micronuclei. There was considerable variation from group to group, but no regular patterns during the cycle. Such variation has been found before for RNA in the macronucleus and cytoplasm and may result from uncontrolled variations in the labeling procedure. It is quite certain, however, that there is no relation between the pattern of RNA synthesis and the pattern of mutation induction during the cell cycle.

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1.4 VARIATIONS IN CELL MASS AND DNA CONTENT WITHIN CLONES OF *PARAMECIUM AURELIA*

R. F. Kimball Stella W. Perdue
E. G. Bailiff

It was established sometime ago[1] that the macronuclear DNA content in the ciliated protozoan *Paramecium aurelia* varies significantly within a clone, probably as a result of imprecise division of the highly polyploid macronucleus. Whether concomitant variations in total cell dry mass also occurred was not certain. The installation of accurate scanning microinterferometric and microspectrophotometric equipment has made it possible to examine this question as part of the broader problems of the control of cell size and of the variation of cell characteristics within a clone.

The first experiment, now completed, involved the determination of the dry mass (microinterferometry) and macronuclear DNA content (Feulgen microspectrophotometry) of a series of different pairs of recently divided sister cells from several different subclones within a clone. The data show that both dry mass and DNA content vary within a clone. The more closely related the cells, the more alike they are. This shows that the variations persist for several cell generations at least, for, if variations from the norm were restored within a single cell generation, the previous ancestry of the cell should have no effect. There is a statistically significant positive correlation between cell mass and DNA content, suggesting that cell mass is dependent, at least in part, on DNA content. The ratio between DNA content and dry mass shows significant variations, however, suggesting that other factors besides the DNA content are responsible for the variations in mass.

The data also serve as a check on the accuracy of our measuring procedures. The smallest variation between measured objects, that between sister cells, furnishes a measure of the upper limit of instrumental error. These coefficients of variation calculated from the sister cell data are very nearly the same but slightly less than those obtained earlier with this same organism in Professor Caspersson's laboratory in Stockholm, suggesting that our instruments are performing satisfactorily as far as repeatability goes. Still smaller coefficients of variation are obtained if the correlation between the microspectrophotometric and microinterferometric data are used to eliminate some of the remaining biological variations.

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1.5 AGE EFFECTS ON MUTATION RATE IN *PARAMECIUM*

Satomi Igarashi

The earlier work revealed the facts that some fraction of x-ray-irradiated paramecia were temperature sensitive at the first postirradiation autogamy,[1] and also that some, not all, temperature-sensitive clones were true mutations which segregated as ordinary recessive traits at the F_2 generation.[2] At the same time, some unknown factors controlling the mutation rate were recognized.

Aging phenomena have been well established in paramecia,[3] especially in syngen 4, stock 51. Abnormal behavior of chromosomes and cytoplasmic changes were reported to increase with increasing age after autogamy. Gradual changes of physiological condition with age suggested that age-dependent susceptibility to x ray and also the effect of age at the first postirradiation autogamy should be examined.

P. aurelia, syngen 4, stock 51, mating type 7, kk-sensitive, was irradiated with x rays of about 2.5 kr during preduplication interphase (G_1), 1.5 hr after previous fission. Isolation of irradiated animals and detection of both mutations, lethal and temperature-sensitive mutation, were performed as previously.

Age-Dependent Mutation Rate. – In the first experiment, dividing animals were collected at various dates after previous autogamy and irradiated. The interfission stage should be the same as G_1 because of the synchrony of sample population. With this method, it became clear that the shorter the time since the previous autogamy the lower the temperature-insensitive mutation rate. The temperature-sensitive lethal mutation rate showed reverse relation in that the less the time

since the previous autogamy the higher the mutation rate. From this experiment, it can be concluded that the measured mutation rate is affected by the age of the clone in such a way that there is a shift from the temperature-sensitive lethal class to the temperature-insensitive lethal class with increasing age.

Age Effect After Irradiation. – In the second experiment, sample populations were kept by daily isolation culture for desired dates after irradiation. The first postirradiation autogamies were induced at 4, 6, 8, 10, and 12 days from the same originally irradiated animals. This method can reveal the age effect on mutation rate within the same population. It became clear that the earlier the autogamous date after irradiation the lower the lethal mutation and the higher the temperature-sensitive mutation.

These two experiments showed that the effect of x-ray irradiation was combined with aging effect in two ways, and this effect was so extensive that mutation date should be compared with special precaution about the age prior to and after irradiation.

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1.6 ULTRAVIOLET-INDUCED MUTATION AND PHOTOREACTIVATION OF MUTATION IN *ESCHERICHIA COLI*

Jane K. Setlow M. E. Boling

Introduction. – These studies were begun for two purposes: (1) to try to find out something about the mechanism of photoreactivation of a particular type of mutation, and (2) to gain information about the mechanism of the mutation (reversion to prototrophy in an arginine-requiring strain). There are two types of photoreactivation which have been described:[1] a direct type, in which pyrimidine dimers are monomerized by a photoreactivating enzyme in the presence of visible light, with resulting higher survival of the bacteria, and an indirect type, in which the visible light causes a growth delay, so that more dark repair can take place. The action spectrum for direct photoreactivation alone was previously obtained with an in vitro system,[2] in which the transforming ability of ultraviolet-inactivated *Hemophilus influenzae* transforming DNA was increased by illumination in the presence of an extract from *E. coli.* Indirect photoreactivation of mutation was measured by illuminating the bacteria with visible light *before* ultraviolet irradiation (photoprotection) and counting the number of resulting mutants. For measurement of total photoreactivation of mutation (direct plus indirect), the bacteria were treated with photoreactivating light *after* ultraviolet irradiation. An action spectrum for induction of the mutation by ultraviolet radiation was obtained in the hope that its shape might suggest what the premutational lesion is.

One possible mechanism of mutation is the conversion of ultraviolet-induced cytosine dimers to uracil dimers by heat, followed by monomerization of the uracil dimers by photoreactivating enzyme.[3] An attempt was made to demonstrate such a mechanism in the case of the mutation to prototrophy by heating the bacteria either before or after photoreactivation. As a control, similar treatment was given to bacteria which lacked the capacity for direct enzymatic photoreactivation, although otherwise the same as the photoreactivable strain used.

Results and Discussion. – We would expect the action spectrum for total photoreactivation of mutation to represent the sum of direct photoreactivation (from the in vitro system) and indirect photoreactivation (obtained from the photoprotection experiments). At the longer wavelengths this appears to be the case. However, the total photoreactivation spectrum has a third peak at about 3250 A, which is a departure from the sum of the other two, suggesting that there is another type of photoreactivation besides the direct and photoprotection types, possibly another indirect type which is only effective when photoreactivating light is administered after ultraviolet irradiation.

The action spectrum for mutation induction falls steadily from its peak near 2600 A down to 2250 A

(the shortest wavelength used), unlike the absorption of DNA or any of its components. Possibly there is a preponderance of lesions induced at the lower wavelengths, which are more likely to be lethal than mutagenic. In any case the action spectrum offers no clue to the nature of the premutational lesions.

The experiments with heat and photoreactivation were suggestive but not conclusive. There was considerably more mutation when the bacteria were heated before photoreactivation rather than after, but heating before and after photoreactivation had no differential effect in bacteria which lack the ability to monomerize dimers. This is just what would be expected if the mutation was caused by cytosine dimerization, with subsequent heat deamination to uracil dimers, followed by monomerization of the latter. Heat alone, however, caused a large reduction in the number of mutations, and thus it is not clear whether the suggested mechanism is the correct one.

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1.7 ULTRAVIOLET INACTIVATION OF *BACILLUS SUBTILIS* TRANSFORMING DNA

M. E. Boling Jane K. Setlow

Introduction. – The first evidence that ultraviolet-induced thymine dimers in DNA are responsible for some of the biological effects of ultraviolet radiation was the discovery that *Hemophilus influenzae* transforming DNA irradiated with a large dose of 2805-A radiation was *reactivated* by a subsequent dose of 2390 A.[1] The number of thymine dimers induced by the 2805-A radiation in this DNA is similarly reduced by the shorter wavelength.[1] Haug and Goes have reported that *B. subtilis* transforming DNA fails to show this short-wavelength reactivation,[2] and they concluded from this and from their action spectrum for inactivation of transforming ability that *B. subtilis* inactivates as a result of damage in cytosine. It is known that two of the possible ultraviolet-induced changes in cytosine (the cytosine-cytosine and thymine-cytosine dimers) are reversible by short wavelength.[3] Furthermore, *B. subtilis* DNA has almost as high an A-T content as *H. influenzae* DNA, and therefore we would not expect the ratio of different types of lesions to differ appreciably in the two DNA's. To determine whether these DNA's really have different responses to ultraviolet radiation, we have obtained an action spectrum for inactivation of the transforming ability of *B. subtilis* DNA, and we have measured the transforming activity after various large doses of long-wavelength followed by short-wavelength ultraviolet irradiation.

Results and Discussion. – The action spectrum we have obtained is considerably different from that of Haug and Goes.[2] The difference could be the result of two differences in method: (1) they used very enormous doses, and (2) they considered the inactivation curves to be the sum of two straight lines on a semilogarithmic plot, whereas their data much more closely fit one straight line when regraphed on a square-root plot. Our action spectrum is similar to that of *H. influenzae* transforming DNA and has a considerably deeper minimum (at about 2350 A) than the absorption spectrum of the same *B. subtilis* DNA (with a minimum at 2300 A), suggesting that damage in thymine is also important in inactivation of this DNA. Furthermore, we obtained short-wavelength reactivation similar to that found in *H. influenzae* DNA, after doses of 2850-A radiation from 1 to 3×10^4 ergs/mm^2. Haug and Goes used doses at this wavelength more than ten times higher, and their failure to obtain short-wavelength reactivation may have been the result of other classes of damage which begin to appear at such enormous doses.

We conclude that *B. subtilis* DNA and *H. influenzae* DNA inactivate by similar mechanisms.

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1.8 FURTHER PURIFICATION OF THE YEAST PHOTOREACTIVATING ENZYME

Amir Muhammed

Purification of the yeast photoreactivating enzyme has been continued with the objective of obtaining a pure enzyme preparation which behaves as a single protein by various physicochemical criteria, such as analytical ultracentrifugation and electrophoresis. After the enzyme is purified by $(NH_4)_2SO_4$ precipitation, Sephadex filtration, $Ca_3(PO_4)_2$ gel absorption and elution, and DEAE chromatography,[1] the final extract is concentrated by dialyzing against saturated Carbowax solution. The concentrated extract is adjusted to pH 7.0 and chromatographed on a hydroxylapatite column by using a gradient of 0.1–0.3 *M* phosphate buffer, pH 7.5. The enzyme elutes at a phosphate concentration of 0.23 *M*. A pink-colored cytochrome band is also eluted along with the enzyme, although it peaks at a phosphate concentration between 0.23 and 0.24 *M*. Further washing of the column with higher concentrations of phosphate buffer does not elute any more enzyme up to 0.33 *M* K_2HPO_4. The enzyme in this second peak is quite unstable as compared to the first. However, the enzyme in both the peaks apparently binds to cytosine dimers as demonstrated by competition experiment with ultraviolet-irradiated poly dI-dC. The enzyme in the first peak is concentrated by dialyzing against Carbowax and then filtered through a Bio-gel P-150 column. The final purification obtained is approximately 5000-fold over the crude extract. The partially purified enzyme is stable in the deep freeze, when stored at pH 7.5–8.5, for at least one month.

The Bio-gel P-150 purified enzyme shows a characteristic fluorescence spectrum for a flavin nucleotide and also a small amount of absorption at 415, 520, and 550 mμ, which is characteristic of a cytochrome. However, it is possible that the cytochrome is a contaminant and might be removed on further purification.

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1.9 THERMAL CROSS SECTION FOR THE REACTION $^{33}S(n,p)^{33}P$

C. M. Steinberg R. C. von Borstel
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S. A. Reynolds[2]

Since the reaction $^{33}S(n,p)^{33}P$ is exothermic in nature, the possibility exists for carrying out the reaction with *thermal* neutrons. This is in contradistinction to the analogous reaction $^{32}S(n,p)^{32}P$, which is endothermic in nature and therefore will occur only with high-energy neutrons. This difference could provide a method for preparing the isotope ^{33}P free from ^{32}P by starting from sulfur only moderately enriched in ^{33}S. The feasibility of the method is dependent, among other things, on the cross section of the reaction $^{33}S(n,p)^{33}P$ to thermal neutrons. Previous reports[3] have yielded highly conflicting values for this cross section.

Two samples of 2 mg each of sulfur enriched to 11.56% ^{33}S were placed in two different positions in the CP-5 reactor at the Argonne National Laboratory. This reactor is moderated with deuterium oxide (D_2O) and is capable of giving better thermalized neutrons at a reasonable flux than the water-moderated reactors in Oak Ridge. Sample No. 1 was placed in the deuterium oxide zone close to but outside the reactor core. Sample No. 2 was placed in the layer of graphite outside the D_2O zone. The samples were exposed for 48 hr.

Radiochemical analysis showed:

Sample	^{33}P (dis min^{-1} mg^{-1})	^{32}P (dis min^{-1} mg^{-1})	Flux (neutrons cm^{-2} sec^{-1})
1	$(9 \pm 1) \times 10^5$	$(1.4 \pm 0.2) \times 10^5$	2.25×10^{13}
2	$(9 \pm 2) \times 10^4$	$(4 \pm 1) \times 10^3$	2.6×10^{12}

These data permit an estimate of the $^{33}S(n,p)^{33}P$ cross section as 6 ± 1 mb. The proportionality between ^{33}P production and total neutron flux indicates that thermal neutrons are indeed responsible for the ^{33}P production, since the "contamination" by high-energy neutrons is much less in sample 2 than in sample 1.

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1.10 PRODUCTION OF ^{33}P FROM SULFUR

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The results of the cross-section measurements discussed in the preceding report ["Thermal Cross Section for the Reaction $^{33}S(n,p)^{33}P$"] indicated that production of ^{33}P in millicurie quantities, free from objectionable amounts of ^{32}P, would be feasible if sulfur somewhat more highly enriched in ^{33}S were available. Improvements in electromagnetic separation techniques enabled the production of sulfur enriched to 68.1% in ^{33}S.

Fifty milligrams of this material was placed in the CP-5 reactor in the same position as sample No. 1 of the preceding report and left for 47 days. About 0.6 mc of ^{33}P (containing <1% ^{32}P) was recovered. While the purity was about as expected, the amount of ^{33}P obtained was less than would be predicted by our cross-section measurements. The cause of this discrepancy is being investigated. Nevertheless, the amount of ^{33}P recovered was sufficient for use in biological experiments. Furthermore, we feel that we have demonstrated the feasibility of producing ^{33}P from ^{33}S.

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1.11 CHANGE OF CHROMOSOMAL SENSITIVITY IN G_1 CELLS

Sheldon Wolff H. E. Luippold

When cells are irradiated in different portions of the cell cycle, different types of chromosomal aberrations are induced. Chromosome aberrations are formed in G_1, whereas chromatid aberrations are produced in S and G_2, and half chromatid aberrations in metaphase. In general, G_1 has been found to be uniformly sensitive to radiation in that, if cells in most portions of G_1 are irradiated, the yields of aberrations are the same. A transition from chromosome to chromatid aberrations occurs in very late G_1. From that point on, the yields of aberrations per unit dose strongly depend on where the cell is in the cell cycle.

This change in apparent sensitivity is not caused by a change in the breakability of the chromosome strands but by a change in the geometric relations of the chromosomes within the interphase nucleus that now enhances the types of rejoining that result in aberrations. Such geometric changes might be expected to occur in the latter part of G_1 (before chromatid aberrations are produced). If they do occur, then cells in these latter portions of G_1 should be more sensitive to radiation.

Soaked seeds of *Vicia faba* were irradiated with 600 r of 250-kvp x rays and then germinated. Some root tips were sampled at the end of 24 hr when the very first divisions were occurring in some (but not all) roots. Other fixations were made 24 hr later when cells from the middle G_1 were at metaphase. Cells that were dividing at the end of 24 hr had occasional chromatid aberrations, indicating that in the soaked seeds there is very late G_1 or early S. In addition, metaphase cells appeared that had only chromosome aberrations. Since these cells were among the first to divide, they were in late G_1 when irradiated. The yields of chromosome

aberrations in these late G_1 cells were 50–100% greater than the yields obtained from cells in earlier portions of G_1.

It therefore appears that, although the sensitivity of G_1 cells is fairly uniform, at the end of G_1, even before the chromosomes react as though double to radiation, there is a shift in the geometric relations within the nucleus that results in an increase in sensitivity.

1.12 ISOLATION AND STUDIES OF MAMMALIAN METAPHASE CHROMOSOMES

J. E. Trosko J. G. Brewen

In order to analyze chromosomes chemically and physically, methods have to be developed to isolate large quantities of unfixed chromosomes. Using 5-aminouracil to partially synchronize Chinese hamster tissue culture cells, we were able to obtain many metaphase cells (metaphase index about 75% after 3 hr of colchicine treatment). After harvesting the cells, a detergent (Nonidet-p40) was used to remove the cell membrane. The detergent treatment was performed over a discontinuous sucrose gradient (0.15 *M* – 0.20 *M* – 0.25 *M*), and immediately after the membranes were removed the chromosome complements were centrifuged at 500 × *g* for 15 min. The pellet contained only nuclei and metaphase chromosome clumps.

Calcium chloride (10^{-4} *M*) was needed to prevent the chromosomes from dissolving. This treatment, however, prevented the isolation of individual chromosomes and resulted in the isolation of complete metaphase plates embedded in cytoplasm or the mitotic spindle. None of the classical reagents (i.e., mercapotethanol, urea, digitonin, arginine) disrupted the metaphase clumps into individual chromosomes. The chromosomes, however, became unraveled after urea and digitonin treatment. Treatment with DNase or trypsin completely disrupted the integrity of the chromosome structure.

Studies are under way in which the isolated metaphase complements are being treated with chelating agents, proteolytic enzymes, DNase, and RNase in an attempt to clarify the structure of the metaphase chromosome. Preliminary results show that after treatment with chelating agents the chromosomes uncoil and demonstrate a multistranded structure with "lampbrush-like" loops.

1.13 MITOTIC SYNCHRONIZATION WITH 5-AMINOURACIL

K. M. Jakob

When the *Vicia faba* root meristem is treated for 24 hr with 5-aminouracil (5AU), the mitotic index drops from 10 to 0 within 8 hr.[1] A burst of synchronously dividing cells (40% mitotic index) may be observed 12–14 hr after removal from the 5AU.[1] A DNA synthesis block had been suspected to be responsible for the synchronization.[1]

Earlier reports[2] had shown that DNA synthesis, as demonstrated by autoradiographs, continued in the presence of 5AU, and that the number of cells in the DNA synthetic portion of the cell cycle (S) increased while the roots were treated with 5AU. On the other hand, continuous ^{3}H-thymidine labeling experiments in the presence of 5AU (ref. 3) indicated that the cells in 5AU enter S at a normal rate.

Results and Discussion. – Pulse-labeling experiments with ^{3}H-thymidine during continuous 19-hr 5AU treatment showed that the number of labeled cells increased during the first 2–6 hr and then reached a plateau 25–40% higher than the control. This increase in number of labeled cells during 5AU treatment is accompanied by a significant decrease in the number of silver grains per nucleus, which returns to normal only about 9 hr after removal from the 5AU.

These findings show that DNA synthesis in 5AU-treated meristematic cells is slowed down and that 500–750 ppm of 5AU extends the time that these cells spend in S by at least 25–40%. This suggests an extension of S from the normal 7.5 hr to at least 11 hr.

Since a reduction in the rate of DNA synthesis per se could not be responsible for the mitotic synchronization 12–14 hr after removal from 5AU, it was of interest to see whether this substance, by its presence in the medium, acts as an agent which completely blocks and accumulates meristematic cells in any portion of the cell cycle until the compound is removed. That this is not so could be seen from continuous 5AU treatments (up to 56 hr) during which mitotic peaks could be observed.[4] Furthermore, a 2-hr treatment with 1500 ppm of 5AU resulted in a drop in the mitotic index 3 hr after removal from 5AU and a 40% mitotic index 12–15 hr after removal from 5AU. This indicated that 5AU does not synchronize by accumulating cells during treatment.

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1.14 STUDIES OF THE REVERSAL OF FUdR-INDUCED DNA SYNTHESIS INHIBITION BY THYMIDINE

Sandra Bell Sheldon Wolff

FUdR (5-fluorouracil deoxyriboside) has been reported by several researchers to break chromosomes of *Vicia faba*. It will also inhibit DNA synthesis in *V. faba*, *E. coli*, and other organisms.[1–3]

In *E. coli*, FUdR inhibits DNA synthesis by inactivating the enzyme, thymidylate synthetase, that converts deoxyuridylate to thymidylate. The inhibition of DNA synthesis is reversed by the addition of exogenous thymine.[2]

In *Vicia faba* there is an apparent reversal of DNA synthesis inhibition by the addition of thymidine.[4] This has been measured by grain counts on autoradiographs. These grain counts have suggested that equal amounts of the ^{3}H-thymidine were incorporated in both FUdR-treated and control roots, even when the concentration of exogenous thymidine was very low.

Experiments were performed to measure DNA synthesis with another label, deoxycytidine, rather than thymidine which, of course, reverses the effect of FUdR.

Results. – In experiments in which lateral roots were treated with solutions containing FUdR, uridine, various concentrations of thymidine, and ^{3}H-deoxycytidine, it was found that there is very little incorporation of the ^{3}H-deoxycytidine in FUdR-treated roots even in the presence of high concentrations of thymidine, that is, even when the inhibition of DNA synthesis by FUdR has been circumvented. This is in contrast to the results of experiments with ^{3}H-thymidine used as the label.

Discussion. – The two most likely explanations for these results are that (1) deoxycytidine inhibits DNA synthesis in the presence of FUdR, and (2) FUdR stimulates the production of endogenous deoxycytidine so that the ^{3}H-deoxycytidine is a smaller percentage of the deoxycytidine present for DNA synthesis in the FUdR-treated roots than in the control roots. Since the mechanism of action of FUdR is the inhibition of the enzyme that converts the deoxyuridylic acid to thymidylic acid and since deoxycytidylic acid can be made from deoxyuridylic acid,[5] any block in the conversion of deoxyuridylic acid to thymidylic acid could possibly increase the pool of deoxyuridylate and thus that of deoxycytidylate.

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1.15 REPAIR OF CHROMOSOMAL DAMAGE IN SEEDS WHICH ARE NOT METABOLIZING

John A. Heddle

By means of fractionation experiments and specific metabolic inhibitors, Wolff has shown that the repair of chromosomal damage is dependant upon metabolic energy and protein synthesis. It would be expected, therefore, that in dry seed, in which the metabolic rate is very low, there would be little or no such repair. Although this has been suggested in the literature, no pertinent data have been published. Since this is an important assumption in some recent work, these experiments were begun to determine whether or not this assumption was justified.

Dry seeds of *Vicia faba* were irradiated at 1 kr/min to give 0, 1, 2, 3, 4, 5, and 6 kr of 250-kvp x rays. The seeds were germinated immediately and allowed to grow until the first mitoses were occurring in the primary root. The roots were then placed in aerated colchicine for 4 hr, fixed, and used to make smear preparations. It was found by means of regression analyses of the frequency of dicentrics and rings observed that these two-break aberrations increase as the square of the dose, with no linear (one-hit) component. This means that all two-break aberrations are induced by a two-hit mechanism. Since this is so, repair of chromosome breaks (induced by the first half of a fractionated dose) before the second half dose is given will reduce the frequency of two-break aberrations by one-half from the frequency produced by the nonfractionated dose. It was necessary to demonstrate a two-hit induction process, for otherwise a failure to demonstrate a fractionation effect could be due either to a lack of repair or to a one-hit process of induction. Since a two-hit dose curve was demonstrated, however, fractionation experiments could be performed and the results interpreted in light of current theory as demonstrating the presence or absence of repair in dry seed.

In the fractionation experiments, a total dose of 4 kr was delivered in two equal doses separated by 1 hr or one, two, or nine days. The seeds were germinated immediately after the second dose had been given. Control groups included 0 kr, 4 kr, 2 kr germinated immediately, and 2 kr stored for nine days before germination. The results of this experiment show that there is a reduction in the frequency of dicentrics and rings when the dose is fractionated, indicating that there is a repair of chromosomal damage in these seeds. The results are complicated by an effect known as the "storage effect," which is the increase in damage that occurs in seeds which are stored for various periods of time between irradiation and germination. This was not expected in these seeds because their moisture content had been found to be 10%, whereas the storage effect had been observed in barley only in seeds having less than 8% moisture. Further experiments are being performed which will determine the degree to which this factor is affecting the fractionation experiments.

1.16 COMPARISON OF THE EXTENT OF THE FUNCTIONAL INACTIVATION IN X-RAY-INDUCED *ad-3*^{R} AND *ad-3*^{IR} MUTATIONS IN *NEUROSPORA*

F. J. de Serres

Studies on the genetic effects of x irradiation in a dikaryon of *Neurospora* with regard to mutation induction have shown that two different classes of mutations are induced in the *ad-3* region: (1) reparable mutants (*ad-3*^{R}) that show one-hit kinetics and (2) irreparable mutants (*ad-3*^{IR}) that show two-hit kinetics.[1,2]

Mutations of both types are being analyzed in a series of complementation tests with allelic or closely linked nonallelic markers as discussed in a previous report.[3] The recent discovery of several *ad-3*^{R} + *RL* mutations induced by ICR-170 (ref. 4), where the *RL* damage is very closely linked to the *ad-3A* or *ad-3B* cistron, as well as marked strains carrying biochemical markers at closely linked loci, has made it possible to distinguish between these two classes of *ad-3* mutations and to map individual strains more precisely.

The results of the complementation or homology tests involving these new testers are shown in Fig. 1.16.1.

The new testers make it possible to demonstrate more clearly the difference between the extent of the functional inactivation in *ad-3*^{R} and *ad-3*^{IR} mutations. Some differences were already apparent in the tests reported previously.[3] Furthermore, the use of biochemical markers at closely linked loci has made it possible to orient the sites of *RL* damage in the different testers, with regard to known biochemical loci in this region of the chromosome, and to demonstrate that the functional inactivation in many mutants extends several map units to the left and/or right of the *ad-3* region.

Tester 93 is particularly useful in the tests on *ad-3A* mutations since it shows homology with all *ad-3A*^{IR} mutations but none of the *ad-3A*^{R} mutations. It has not been possible to localize the site of *RL* damage in this tester, precisely, in these tests, but it is possible that homology tests with (*ad-3A ad-3B*)^{IR} double mutants will make it possible to tell whether this site is to the left or

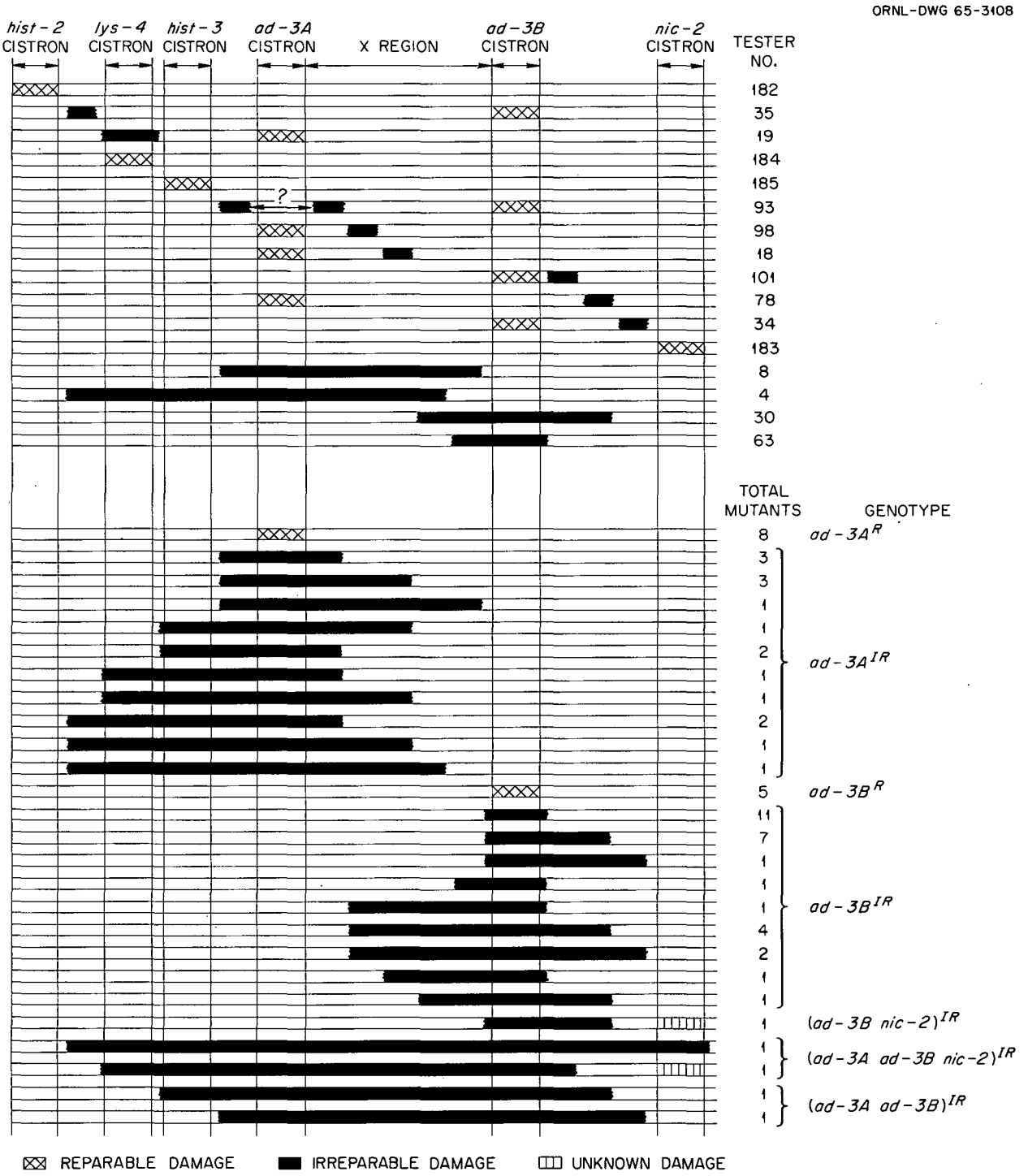

Fig. 1.16.1. Complementation Map of the *ad-3* and Immediately Adjacent Regions Showing the Tester Set Used in Homology Tests and the Extent and Type of Functional Inactivation in Various Types of *ad-3* Mutations Induced in a Dikaryon.

to the right of the *ad-3A* cistron. The use of tester 101 has shown that the functional inactivation in $^{8}/_{19}$ *ad-3B*^{IR} mutations (that were indistinguishable from *ad-3*^{R} mutations in tests presented previously[3]) extends beyond the boundaries of the *ad-3B* cistron to the right.

The complexity of the genetic alterations in some of the mutations testing as either (*ad-3B nic-2*)^{IR} or (*ad-3A ad-3B nic-2*)^{IR} is quite striking. The functional inactivation in some such mutations appears to be discontinuous, in contrast to the continuity that might have been expected. It appears that two of these have resulted from chromosome deletions of the *ad-3A* and/or the *ad-3B* locus, in addition to a separate site of genetic damage affecting the *nic-2* locus. The precise nature of the damage affecting the *nic-2* locus is unknown.

It is also of interest that the sites of *RL* damage in testers 98 and 18 do not show homology. If the *RL* damage in tester 93 is to the left of the *ad-3A* cistron, then the present tests provide evidence for at least four different functional units in the X region between the *ad-3A* and *ad-3B* cistrons.

Although much has been accomplished in a relatively short time, it is clear that additional tester strains must be sought with sites of *RL* damage closer to the *ad-3B* cistron. Even in the present tests, $^{11}/_{29}$ *ad-3B*^{IR} mutations are indistinguishable from *ad-3B*^{R} mutations. The results of tests on the *ad-3A* mutations suggest that this is most likely due to the inadequacy of the present tester set for mutations affecting the *ad-3B* cistron.

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1.17 LOCALIZATION AND IDENTIFICATION OF THE GENETIC ALTERATIONS IN *ad-3*^{IR} MUTATIONS INDUCED BY ICR-170

F. J. de Serres H. E. Brockman

Recent studies on the mutants produced by ICR-170 indicate that this compound produces predominantly intragenic alterations in the *ad-3* region of *Neurospora*.[1,2] In a sample of 187 ICR-170–induced mutants, 57 were *ad-3A* and 130 were *ad-3B*. No (*ad-3A ad-3B*) double mutants were found, in contrast to the large number found in an identical experiment with x irradiation.[3,4] Furthermore $^{150}/_{187}$ mutants were actually *ad-3* + *RL* (where *RL* designates a separate site of recessive lethal damage elsewhere in the genome). Tests for allelic complementation showed that 65% of the complementing *ad-3B* mutants have polarized complementation patterns, suggesting that the major class of genetic alterations produced by this mutagen is base-pair addition and/or deletion.[2] Because of these correlations it seemed highly likely that $^{9}/_{187}$ *ad-3* mutations that tested as either *ad-3A*^{IR} or *ad-3B*^{IR} in homology tests with tester strain 12-1-18 (ref. 5) might actually be *ad-3*^{R} + *RL* mutations (where *RL* designates a separate site of genetic damage closely linked to the *ad-3* region). A priori, this high percentage of mutants with an additional genetic alteration in the immediate vicinity of the *ad-3* region is unexpected but might be correlated with the unusual mutagenic activity of ICR-170.

In addition, *ad-3*^{R} + *RL* mutations with the separate site of genetic damage closely linked to the *ad-3* region have been particularly useful as tester strains in homology tests with *ad-3*^{R} and *ad-3*^{IR} mutations discussed in a previous report,[5] and additional tester strains are needed to try to determine the difference in the extent of the functional inactivation in these two classes of x-ray-induced *ad-3* mutations.

An attempt to determine the full extent and type of genetic damage in each of these nine mutations was made by a series of homology tests with three irreparable mutants (with more restricted functional inactivation than that found in strain 12-1-18) as testers. These were (1) 12-6-161 – (*ad-3A ad-3B*)^{IR}, (2) 12-7-215 – *ad-3A*^{IR}, and (3) 12-5-182 – *ad-3B*^{IR}. Only three of the nine mutations showed homology with 12-6-141, showing that $^{6}/_{9}$ were actually

ad-3^R + *RL* mutations and suggesting that, if the other three were of the same type, the *RL* damage was very closely linked to the *ad-3* region. This latter group of three mutations was analyzed in a series of very extensive homology tests with 16 *ad-3A*^{IR}, 10 *ad-3A*^{R} + *RL*, 29 *ad-3B*^{IR}, 7 *ad-3B*^{R} + *RL*, 1 (*ad-3B nic-2*)^{IR}, 2 (*ad-3A ad-3B*)^{IR}, and 2 (*ad-3A ad-3B nic-2*)^{IR} mutations. The results of this analysis in relation to previous homology tests[5] on some of these strains are illustrated in Fig. 1.16.1.

This series of homology tests showed conclusively that each of the three ICR-170–induced mutations (93, 98, 101) is *ad-3*^{R} + *RL* rather than *ad-3*^{IR}. In each case the separate site of *RL* damage is very closely linked either to the *ad-3A* or *ad-3B* cistron. It is of particular interest that the *RL* damage in tester 98 is in the X region and that this damage is not identical with that found in tester 18. The *RL* damage in tester 101 is very closely linked with the *ad-3B* cistron on the right. The *RL* damage in tester 93, however, is homologous to that found in all *ad-3A*^{IR} mutations, but it is not homologous to that found in any of the *ad-3A*^{R} or *ad-3B*^{IR} mutations. Thus it is impossible to determine from current data whether it is closely linked to the *ad-3A* cistron on the left or on the right.

The present analysis shows that $^9/_{187}$ *ad-3* mutants induced by ICR-170 and classified as *ad-3*^{IR} mutations on the basis of homology tests with tester 12-1-18 are actually *ad-3*^{R} mutations resulting from intragenic alterations in the *ad-3A* or *ad-3B* locus. In this respect the spectrum of genetic effects produced by this compound is more closely related to that of nitrous acid[6] than to x irradiation.[3,4]

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1.18 IDENTIFICATION OF THE MOLECULAR PART OF THE ACRIDINE MUSTARD (ICR-170) IMPORTANT FOR ITS MUTAGENICITY[1]

H. V. Malling

A monofunctional nitrogen mustard derivative of acridine (ICR-170; Fig. 1.18.1) has been shown to be a potent antineoplastic compound against ascites tumors in mice. Creech *et al.*[2] have synthesized ICR-170 and many related compounds and tested these for their antineoplastic activity. By comparative studies of these compounds' antineoplastic activity, it was found that both the acridine ring and the alkylating part of the ICR-170 molecule are important for their activity.

Brockman[3] has shown that ICR-170 is highly mutagenic for *Neurospora* at a very low lethality, and, by comparative studies of the leakiness and complementation of the *ad-3* mutants induced by ICR-170 and mutants induced by 2-aminopurine, x rays, and nitrous acid, he suggested two alternative mechanisms for the mutagenicity of ICR-170:

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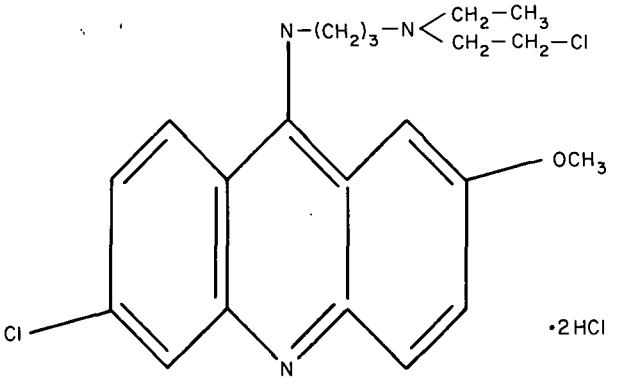

Fig. 1.18.1. ICR-170: 2-Methoxy-6-chloro-9-(3-[ethyl-2-chloroethyl]aminopropylamino)acridine Dihydrochloride.

(1) that most of the mutants are base-pair insertions and deletions or (2) that the mutants are predominantly base-pair substitutions of a special kind (nonsense mutations) or mutations localized specifically in those base-pair triplets coding for amino acids essential for the function of the enzyme.

By using ICR-170 and structurally related compounds in a comparative mutagenesis study, it should be possible to identify those parts of the ICR-170 molecule essential for its mutagenicity. This study was done with the tester set previously described;[4] this set consists of four strains which only revert by base-pair substitutions, two strains which only revert by base-pair insertions and/or deletions, and two strains which revert only spontaneously.

Compounds consisting of the aliphatic part of the ICR-170 (the usual monofunctional nitrogen mustard) induced reversions only in the strains that revert by base-pair substitutions. The acridine "Atabrine" which has nearly the same structure as ICR-170, but which is not alkylating, did not induce reversions in any of the tester mutants. A bifunctional acridine mustard with the same ring structure and side chain as ICR-170 was even more mutagenic than ICR-170. Compounds which are composed of the same side chain and the same monofunctional nitrogen mustard as ICR-170, but with only the left two-thirds or the right two-thirds of the acridine ring in ICR-170, are completely inactive even at a molarity two times higher than the one used for ICR-170. This finding suggests that the acridine ring structure has to be intact for the mutagenicity.

It seems likely that the mutagenic activity of ICR-170 depends on alkylating moiety, as well as the acridine ring producing both base-pair substitutions and base-pair insertions and/or deletions respectively. The reversion frequencies of those mutants treated with ICR-170, which only revert by base-pair substitutions, are 10–100 times greater than the reversion frequencies obtained after treatment with the comparable aliphatic nitrogen mustards. This may be explained by the fact that the acridine ring, which combines with great affinity to DNA, functions as a carrier of the alkylating part of the ICR-170 molecule to the DNA. Acridines cannot produce mutations in *Neurospora*. However, it may be that, when the acridine ring is covalently bound to DNA through an alkylation, the acridine may not release from the DNA during replication and in this way may induce base-pair insertion and/or deletion.

Another aspect of this analysis is that it has become apparent that reversions of the mutants used occur only under specific conditions. Therefore they can be used as a tester set to detect particular types of mutagenic activity. They can, therefore, be used: (1) to select compounds that have specific mutagenic activity, for more detailed study of their forward mutation spectra; (2) to test compounds which are doubtful mutagens under many different conditions to determine whether they possess mutagenic activity (for example, to test a correlation between carcinogenic and mutagenic activity of such compounds as the polycyclic hydrocarbons); and (3) to investigate the association between the antineoplastic activity and the mutagenicity.

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1.19 PROTECTIVE EFFECT OF ANOXIA ON X-RAY-INDUCED NUCLEAR INACTIVATION IN *NEUROSPORA*

B. B. Webber F. J. de Serres

Kølmark[1] has previously demonstrated a protective effect of anoxia against both mutagenesis and against conidial inactivation in *Neurospora*. In the present experiment a heterokaryon is used to test whether the inactivation thus protected against is cytoplasmic or nuclear. If killing is predominantly cytoplasmic, then percentage survival of heterokaryotic and homokaryotic conidia should decline equivalently with dose. Nuclear inactivation should be expected to affect homokaryotic and heterokaryotic conidia differently.

Conidia of heterokaryon 12 (ref. 2) were harvested and suspended in water at a concentration of 1.1×10^6/ml and then x irradiated at ~25°C under

either aerobic or anoxic conditions (with a Maxitron 250 source at 250 kvp, 30 ma, with 3-mm aluminum and 11-mm Lucite extrinsic filtration) with doses of 5, 10, 20, or 40 kr at a dose rate of 1000 r/min. Aerobic suspensions were bubbled during exposure and for 5 min before exposure with air. Anoxic cultures were bubbled during exposure and 30 min before exposure with high-purity nitrogen. Conidia were then surface plated on minimal medium to detect heterokaryotic surviving conidia and on a supplemented medium to detect heterokaryotic conidia, as well as those homokaryotic for one of the nuclear components (the component requiring histidine, adenine, nicotinamide, and inositol). In Fig. 1.19.1, survival, expressed as a percentage of the zero-dose survival, is plotted logarithmically against dose. Anoxic protection is indicated by the much faster decrease in survival of conidia which were aerobic when irradiated. The ratio of anoxic dose to aerobic dose required to produce comparable effects gives a dose reduction factor of about 2. Furthermore, under a given condition of irradiation, the heterokaryotic survival decreases more rapidly than does survival of combined heterokaryotic and homokaryotic components. In fact, the plot of homokaryotic conidia alone (colonies on supplemented medium minus colonies on minimal) shows an initial increase in percentage survival as heterokaryotic conidia are converted by nuclear inactivation into homokaryotic conidia.

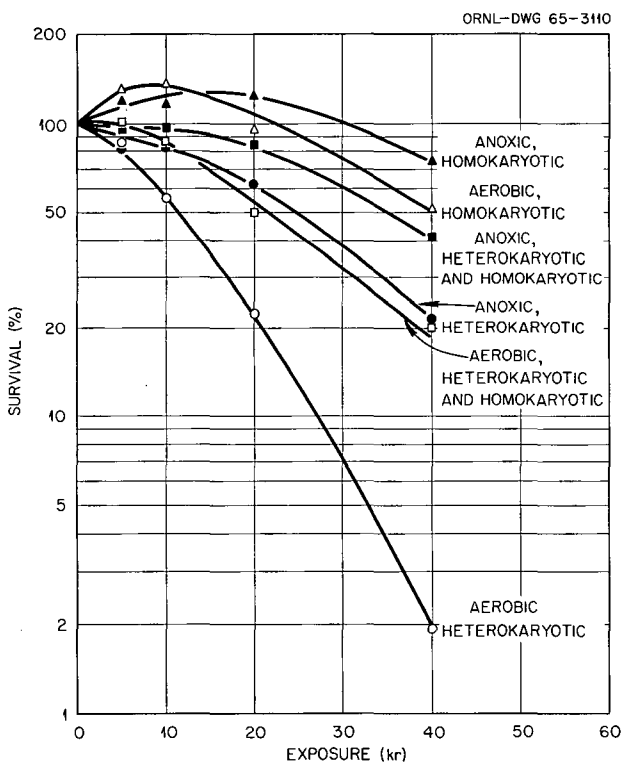

Fig. 1.19.1. Dose-Survival Curves for Conidia X-Irradiated Under Aerobic and Anoxic Conditions.

This study of the effects of anoxia on conidial inactivation in a heterokaryon was designed to determine the most desirable conditions for the induction of forward mutations in the *ad-3* region with x rays, under anoxic conditions. The study of the genetic effects of x irradiation under conditions of anoxia will be comparable to the aerobic studies reported previously.[2–4] If the same dose reduction factor of 2 is found for mutation induction, then it will be possible to determine whether the protective effect of anoxia is exerted equally or differentially against reparable mutants, which are single hit in origin, and irreparable mutants, which are two hit in origin and probably result from chromosome deletion.[5]

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1.20 A STUDY OF THE COMPLEMENTATION AND REVERSION PATTERNS EXHIBITED BY A GROUP OF ULTRAVIOLET-INDUCED *ad-3B* MUTANTS[1]

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

The object of these experiments has been to obtain information concerning the nature of the mutational changes induced by ultraviolet in

Neurospora crassa. Two approaches have been made to this problem: a study of the complementation patterns exhibited by ultraviolet-induced mutants, and a study of their abilities to revert with selected chemical mutagens. It is not unreasonable to suppose that the nature of the original mutagenic lesion will influence its complementation and reversion behavior.

The mutants used in this work were obtained from three forward-mutation experiments using the wild-type strain 74-OR23-1A. The conidia were treated with 2400 or 3000 ergs/mm^2 ultraviolet. Approximately 1500 mutants were isolated by the direct method.[2] The genotype of each isolate was determined in heterokaryon tests with standard *ad-3A* and *ad-3B* tester strains.

Five hundred eighty-six of the *ad-3B* mutants were selected for use in allelic complementation tests. Each was combined, in turn, with a series of 19 *ad-3B* tester mutants, which between them span the entire *ad-3B* complementation map of 17 complementation units. The spectrum of complementation patterns found is summarized in Fig. 1.20.1. The data are presented in the conventional way; mutants with overlapping bars fail to complement each other.

As described previously,[3] the complementation patterns can be grouped into three different classes. These are the noncomplementing mutants, the polarized complementing mutants, and the nonpolarized complementing mutants. The first class is by far the most numerous. Approximately 10% of the *ad-3B* mutants are able to grow to varying extents in the absence of adenine. In every case where they could be tested they proved to be of the nonpolarized complementing type, a finding in close agreement with earlier data of de Serres *et al.*[4]

The three mutagens used in the reversion experiments were nitrous acid (NA), ethyl methanesulfonate (EMS), and an acridine mustard (ICR-170), to permit a direct comparison with data from reversion studies on NA-induced adenine-requiring mutants of *Neurospora* described in a previous report.[5] These mutagens are expected to induce predominantly base-pair substitutions (NA and EMS) and base-pair additions and/or deletions (ICR-170). Rather than attempt to obtain quantitative data for each mutant with each mutagen, 60 were chosen from each of the three groups, and each mutant was screened with each mutagen in rapid semiquantitative tests. The number of mutants in each class responding to the mutagenic treatments is shown in Table 1.20.1. The discrepancies between the selected numbers and the tested numbers arose from leakiness, which had not been sufficient to affect the complementation tests but which invalidated the reversion tests, and from the repeated failure of some mutants to give insufficient conidia.

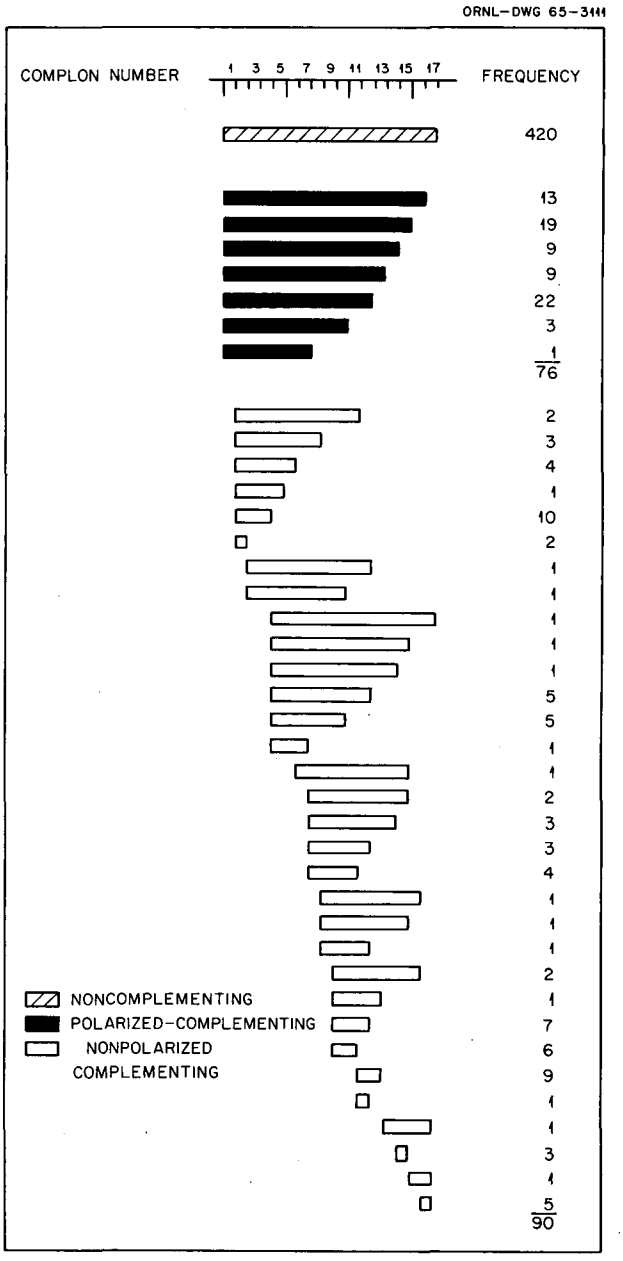

Fig. 1.20.1. Complementation Patterns of Ultraviolet-Induced *ad-3B* Mutants.

Table 1.20.1. The Revertibility of Ultraviolet-Induced *ad-3B* Mutants After Treatment with NA, EMS, or ICR-170

Mutagen			Nonpolarized		Polarized		Noncomplementing	
NA	EMS	ICR-170	Number Responding	Percent	Number Responding	Percent	Number Responding	Percent
0	0	0	13	31.7	20	36.3	23	39.6
0	0	+	3	7.4	16	29.0	21	36.2
0	+	0	3	7.4	5	9.0	0	
0	+	+	4	9.7	1	1.8	1	1.7
+	0	0	4	9.7	1	1.8	6	10.3
+	0	+	1	2.4	3	5.4	2	3.4
+	+	0	6	14.6	4	7.2	4	6.8
+	+	+	7	17.0	5	9.0	1	1.7
		Totals	41		55		58	

If the mutagenic action of these chemicals in *Neurospora* is as believed, $^{25}/_{28}$ of the nonpolarized complementing mutants which revert at all do so with agents causing base-pair substitutions. The assumption is made that base-pair substitutions can only be reverted by further base-pair substitutions. At present we cannot decide whether these result from events at the originally altered site or at a different site. This being the case, the result agrees well with an earlier suggestion of de Serres *et al.*,[3] that mutants which display nonpolarized patterns of complementation are, in all probability, the result of base-pair substitutions. In general, alterations of this type seem less likely to cause such extensive damage to the enzyme concerned as do mutations of the "reading frame" type. Here, extensive alteration or loss of the protein is to be expected. The noncomplementing and polarized complementing mutants might be of this type, and, indeed, they are far more revertible with the acridine mustard alone than are the nonpolarized complementing class. As with the earlier analysis of the NA-induced mutants,[5] it is clear that NA-revertible damage induced by ultraviolet can give rise to noncomplementing and polarized complementing mutants. This may indicate that base-pair substitutions give rise, possibly by "nonsense mutations," to polypeptide fragments[6] of sufficient size to give polarized complementation patterns. A particularly puzzling aspect of the ultraviolet data is the large numbers of mutants which complement but do not revert. An explanation is being sought for this, since the data are in marked contrast to that obtained from an analysis of NA-induced mutants. It will be particularly important to determine whether these mutants do not revert or whether they can only revert after treatment with as yet untested mutagens such as ultraviolet.

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1.21 PATTERN OF DNA SYNTHESIS IN X-AUTOSOME TRANSLOCATIONS IN THE MOUSE

E. H. Y. Chu Liane B. Russell[1]

Introduction. – Recent cytological results in man and other mammals indicate the single nature of sex chromatin and the difference between two X's, both in morphology at late prophase and telophase, and in time of DNA synthesis during interphase. In the mouse, however, the sex chromatin pattern and the timing of DNA synthesis in the X chromosomes have not been demonstrated, although Ohno and Hauschka[2] showed that in late prophase cells of various tissues of female mice, one chromosome (presumably an X) was heteropyknotic. On the basis of this cytologic observation and other genetic data (XO females; mosaic phenotype in females heterozygous for X-linked genes; variegated-type position effect in X-autosome translocations), a hypothesis for genetic inactivation of more than one X chromosome in mammals has been postulated.[3–5] In the mouse, such inactivity does not appear to involve one entire X chromosome, and those parts of the chromosome that can change to an inactive state spread inactivation along a gradient to limited distances.[6] Cytologically, the murine X chromosome remains unidentified, although it has been assumed to be among the longest in the complement. Using translocation stocks between the X chromosome and linkage group 1 (L.G. 1), the present autoradiographic studies reveal small but discrete differences in the timing of DNA synthesis between the two X chromosomes in female mice and should make it possible to identify cytologically the L.G. 1 chromosome, and perhaps the X chromosome.

Materials and Methods. – Two stocks, R2 and R3, carrying X-1 translocations of independent origin and with breakpoints mapped in different locations[6] were used. Females with the balanced reciprocal translocation and heterozygous for autosomal markers were crossed with normal recessive males. The crosses were made in such a way that eye color of the fetuses would distinguish translocation heterozygotes from normal segregants (except for rare crossovers). Fifteen-day-old fetuses were removed aseptically, and the trypsinized cells from each individual fetus were grown in culture. Tritiated thymidine was added by one of two methods: (1) pulse labeling for 15 min during various mitotic stages of 5-aminouracil synchronized cell cultures, or (2) continuous labeling in nonsynchronized cultures. In the latter case, 1-hr colchicine treatment before fixation was given at various times to study the terminal stages of DNA synthesis. Suitable orcein-stained metaphase figures were photographed. The slides were then coated with nuclear track emulsion, exposed, developed, and restained with toluidine blue. The cells previously photographed were relocated and rephotographed.

Results and Conclusions. – 1. In R2 one of the translocated X-1 chromosomes is as long as, or possibly slightly longer than, the longest pair in the complement. The reciprocally translocated chromosome falls within the size range of normal chromosomes and is thus not morphologically identifiable. In R3, one of the translocated chromosomes is longer than the longest pair. The present results on translocation females concern R2 only. The long translocation chromosome will be referred to as the T chromosome. Its presence or absence in different fetuses agreed with expectation from eye color.

2. Sexing at the cellular level is possible because the Y chromosome in certain mouse strains can be identified.[7] In normal males, only the Y chromosome replicated with obvious asynchrony. In males with the translocation, the T chromosome incorporated ^{3}H-thymidine in timing similar to that of the rest of the complement, as was expected.

3. Cells analyzed to date came only from cultures labeled for long intervals. Probably as a result of mixture of cell types with different mitotic cycles (whole fetuses used), cells presumably labeled in different stages of S could be observed. So far, in normal females, there are certain indications of a slightly late replicating chromosome, possibly one of the X's. This differentiation is not as striking as in man, hamsters, cat, cattle, and some other mammals. It cannot yet be said with certainty that this indicates that mice manifest the heterocycly to a smaller degree. Possibly, analysis of cultures labeled for shorter periods will reveal more differential labeling in late S.

4. In females carrying the translocation, the T chromosome in certain cells replicated more or less simultaneously with other chromosomes. Either the T chromosome was in very early S or it was one containing the genetically active X portion.

In other cells, however, differences in labeling were detected in different segments of the T chromosome. During what may be presumed to be early or mid S, the proximal $\frac{2}{3}$ of the T chromosome was lightly labeled, and the distal $\frac{1}{3}$ was very heavily labeled. In some cells, one medium-sized autosome also appeared to be heavily labeled at the distal end, and this could be the intact L.G. 1 chromosome.

In cells presumed to represent somewhat later stages in S, the proximal $\frac{2}{3}$ became more heavily labeled, but both the kinetochore region and the distal $\frac{1}{3}$ became completely unlabeled, indicating completion of DNA synthesis in these regions.

Finally, the proximal $\frac{2}{3}$ appeared slightly more heavily labeled, while other regions of this chromosome and all other chromosomes in the complement were either unlabeled or very lightly labeled. This difference at what may be a late stage of S is interpreted as indicating differential timing of DNA synthesis in the two X's in female mice.

5. At least part of the translocated segment retains its autonomous timing of replication. Genetic evidence indicates a limited influence from X to autosome with respect to gene inactivation. The present cytological evidence establishes the independence of gene replication in the distal autosomal parts of an X-autosome translocation, corroborating the genetic finding[6] of limited spread of inactivation.

6. The reciprocally translocated chromosome was not detectable by asynchronous replication. This, too, corroborates the genetic evidence that indicates X differentiation to be controlled by a point or region in the X.[8] Thus, the segment of the X removed by translocation apparently no longer behaves differentially.

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1.22 RADIATION SENSITIVITY IN SYNCHRONIZED MAMMALIAN CELLS

E. H. Y. Chu

Experiments have been continued on mitotic inhibition, suppression of DNA synthesis, and chromatid aberrations induced by x ray or ultraviolet in 5-aminouracil–synchronized Chinese hamster cells in vitro. Successive samples of the chemically synchronized cells were pulse labeled with ^{3}H-thymidine just prior to x (150 r) or ultraviolet (100 ergs/mm^2 at 2650 A) irradiation every hour for 7 hr. Both the irradiated and nonirradiated cell populations were treated with colchicine for 3 hr at the seventh hour. Additional samples were fixed immediately after each radiation exposure in order to determine the proportion of labeled interphase cells.

Earlier work[1] indicates that there were only a few mitoses during the first 6 hr after removal from the chemical; mitotic figures quickly appeared thereafter, and the frequency of dividing cells reached a peak at the eighth hour. The colchicine treatment accumulated metaphase figures up to 27%. The mitotic indices were reduced to about 7% when cells were ultraviolet-irradiated during various phases of S, and to 13% when irradiated at G_2. There was practically no inhibition of mitosis with x irradiation at the dose used during S, but mitotic activity was drastically reduced (to 4%) when cells were irradiated at G_2.

During the first 3 hr after removal of cells from 5-aminouracil (5AU), almost all the cells (100% metaphase and over 70% interphase) actively engaged in DNA synthesis. An orderly completion of DNA synthesis and normal entry to G_2 and mitosis were seen in both unirradiated and irradiated populations. Both types of radiation, at the doses here employed, did not seriously affect the sequence and timing of cell stages, and only slightly reduced the rate of thymidine incorporation.

With x rays, the frequency of induced isochromatid breaks remained constant during S and G_2, whereas those of chromatid exchanges increased somewhat,

and chromatid terminal deletions increased significantly at G_2. This result agrees with that of Brewen[2] in *Vicia*, which he interpreted as an increase in the number of sites for exchange.

With ultraviolet, the highest frequencies of induced chromatid aberrations, including all types, occurred at early S; the frequencies became much reduced during the remainder of S and were lowest at G_2. The direct action of ultraviolet on DNA suppresses synthesis and reduces the number of cells that can reach mitosis. Although chromosome aberrations can be induced by ultraviolet in all stages of the cell cycle,[3] the present result probably indicates that chromosomes during replication are more vulnerable to ultraviolet irradiation.

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1.23 RADIORESISTANT HUMAN AMNION CELLS

J. D. Regan[1]

Attempts to derive radioresistant variants of human amnion cells are in progress. Sublines of the RA ("recovered amnion") human amnion cell[2] surviving 300, 700, and 1500 r of x rays have been grown out. Clones have been established from survivors of 700 and 1500 r. This cell is of interest due to its near-triploid constitution.[3]

Clone $RAX7C_1$, derived from survivors of 700 r (surviving fraction apparently less than 10^{-6}), irradiated with 800 r, displayed elevated survivorship possibly greater than ten times the wild-type RA cell. This preliminary result seems to indicate a likelihood of developing RA clones having a radiosensitivity clearly distinguishable from the wild-type RA cell. In view of recent studies[4] of ^{3}H-thymidine incorporation after x irradiation in radiosensitive and radioresistant bacteria, this aspect may be of interest in normal and radioresistant human cells. These experiments require a convenient method for assaying ^{3}H-thymidine incorporation without terminating the culture. Thus, cells are grown in suspension on a rotary shake table, aliquots are removed periodically and labeled by incubation in radioactive medium, and the cells are lysed by brief sonication. (For lysing the labeled cells, repeated freeze-thaw cycles were found less than 50% effective; 15-sec sonication apparently lysed all the cells.) Aliquots of the sonicate are placed on filter paper disks, which are then washed according to the method of Bollum.[5] Radioactivity is determined in a liquid scintillation counter.

The genetic basis of radioresistance in human cells is of interest intrinsically and also because of the known development of radioresistance in tumors treated with therapeutic x rays.[6]

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1.24 AMINO ACID SEQUENCE IN α-CHAIN TRYPTIC PEPTIDES OF C57BL MOUSE HEMOGLOBIN

R. A. Popp Diana M. Popp

Introduction. – The sequence of amino acids in the tryptic peptides of the α chain of C57BL hemoglobin is being studied.

Methods and Results. – Edman degradation procedures[1] are being used; the *N*-terminal amino acids are successively removed and identified with the Spinco amino acid analyzer. The larger tryptic peptides were digested with chymotrypsin[2] before their sequence could be studied, because the procedures usually limit determination of sequences over five to seven amino acid residues. To date the sequence has been completed for 11 of the 14 tryptic peptides in the α chain. The sequences are reported in Table 1.24.1, and the

Table 1.24.1. Comparison of the Amino Acid Sequences in the α Chain of Human and Mouse Hemoglobin

							αT-1				αT-2					αT-3	
Mouse	Val.	Leu.	Ser.	Gly.	Glu.	Asp.	Lys.	Ser.	Asn.	Ileu.	Lys.	Ala.	Ala.	Try.	Gly.	Lys.	Ileu.	Gly.
Human	Val.	Leu.	Ser.	Pro.	Ala.	Asp.	Lys.	Thr.	Asn.	Val.	Lys.	Ala.	Ala.	Try.	Gly.	Lys.	Val.	Gly.
							7				11					16		
							↓						αT-4					
Mouse	Gly.	His.	Ala.	Gly.	Glu.	Tyr.	Gly.	Ala.	Glu.	Ala.	Leu.	Glu.	Arg.	Met.	Phe.	Ala.	Ser.	Phe.
Human	Ala.	His.	Ala.	Gly.	Glu.	Tyr.	Gly.	Ala.	Glu.	Ala.	Leu.	Glu.	Arg.	Met.	Phe.	Leu.	Ser.	Phe.
													31					
				αT-5			↓			↓								
Mouse	Pro.	Thr.	Thr.	Lys.	Thr.	Tyr.	Phe.	Pro.	His.	Phe.	Asp.	Val.	Ser.	His.	Gly.	Ser.	Ala.	Glu.
Human	Pro.	Thr.	Thr.	Lys.	Thr.	Tyr.	Phe.	Pro.	His.	Phe.	Asp.	Leu.	Ser.	His.	Gly.	Ser.	Ala.	Glu.
				40														
		αT-6				αT-7	αT-8				αT-10							αT-11
Mouse	Val.	Lys.	Gly.	His.	Gly.	Lys.	Lys.	----------		Leu.	Arg.	Val.	Asp.	Pro.	Val.	Asn.	Phe.	Lys.
Human	Val.	Lys.	Gly.	His.	Gly.	Lys.	Lys.	----------		Leu.	Arg.	Val.	Asp.	Pro.	Val.	Asn.	Phe.	Lys.
		56				60	61				92							99
				αT-14														
Mouse	----------		Tyr.	Arg.														
Human	----------		Tyr.	Arg.														
				141														

NOTE: The residue positions and the tryptic peptide numbers are given below and above each arginine and lysine. Residues showing amino acid differences are indicated by boxes. Peptide bonds in αT-4 and -6 cleaved by chymotrypsin for studying the sequence in these peptides are indicated by arrows.

amino acid sequence for the comparable peptides of human hemoglobin A are shown. The amino acid sequences of αT-9, αT-12, and αT-13 have not been completed.

Discussion. – The sequence of amino acids in the α chain of mouse hemoglobin is homologous to that in human hemoglobin. Six peptides (αT-1, -3, -7, -8, -11, and -14) from mouse and human hemoglobin are identical. The amino acid substitutions noted in the other peptides are generally of a conservative type with respect to probable alterations in the sequence of purine and pyrimidine bases in the triplet code of DNA.

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1.25 LINKAGE OF LOCI CONTROLLING SERUM ESTERASE PATTERNS AND OLIGOSYNDACTYLISM

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

Introduction. – Zymograms of serum esterases of inbred strains of mice show two patterns in the albumin region following starch gel electrophoresis.[1] Linkage tests show that the locus, which controls these esterase patterns, is closely linked to *Os* in linkage group XVIII.

Methods. – Male heterozygous oligosyndactyls, homozygous for the *b* allele at *Es-1* ($OsEs\text{-}1^b/+Es\text{-}1^b$), were crossed to C57BL females ($+Es\text{-}1^a/+Es\text{-}1^a$). F_1 females ($+Es\text{-}1^a/OsEs\text{-}1^b$) were backcrossed to C57BL males. The backcross progeny were visually classified for *Os*, and the serum esterase types were identified by starch gel techniques.[1]

Results and Discussion. – Genotypes of the 163 backcross progeny are presented in Table 1.25.1. Four recombinants were observed; the recombination frequency was 0.0245 ± 0.0121. Other marker genes in linkage group XVIII are tottering,[2] hooked,[3] and sombre.[4] The position of *Es-1* with respect to these loci is as yet unknown.

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1.26 SOME PROPERTIES OF A NON-*H-2* ISOANTIGEN IN RFM MICE

Diana M. Popp R. A. Popp

Introduction. – In C3H anti-RFM serum prepared for the purpose of characterizing RFM *H-2* isoantigens, a red-cell agglutination antibody was found which retained activity for RFM after absorption with A·CA,[1] a strain of similar *H-2* phenotype as

Table 1.25.1. Backcross Progeny Classified for *Os* and *Es-1*

Mating	Offspring				Total
	$\frac{+Es\text{-}1^a}{+Es\text{-}1^a}$	$\frac{OsEs\text{-}1^b}{+Es\text{-}1^a}$	$\frac{+Es\text{-}1^b}{+Es\text{-}1^a}$	$\frac{OsEs\text{-}1^a}{+Es\text{-}1^a}$	
$\frac{+Es\text{-}1^a}{OsEs\text{-}1^b} \times \frac{+Es\text{-}1^a}{+Es\text{-}1^a}$	75	84	2	2	163

RFM (i.e., $H\text{-}2^f$). Various analyses were performed to determine some properties of the antigen.

Methods and Results. – The antiserum was produced by five intraperitoneal injections of RFM liver into strain C3H mice. Erythrocyte agglutinations, in vitro absorptions, and white-cell agglutinations were done by techniques previously described.[2–4] Analysis of (RFM × 101)F_2 and (RFM × C57BL)F_2 mice, tested for their *H-2* phenotype and the unknown antigen, shows independent segregation of the unknown antigen from $H\text{-}2^f$ (Table 1.26.1). Therefore, the antigen is a non-*H-2* antigen. Analysis of other marker genes in these two populations shows no linkage of the non-*H-2* antigen with *A*, *Hb*, *Es-1*, *c*, or sex. In vitro absorption indicated that testis and brain have appreciable quantities of the antigen; these tissues possess little *H-2* antigen. Tail grafts were exchanged among the (RFM × 101)F_2 mice to study the effect, if any, of the antigen on skin grafts. Five grafts as described in Table 1.26.2 were placed on each animal. Results indicate that a difference at the non-*H-2* locus induced graft rejection; the time of rejection averaged seven days longer than rejections caused by *H-2* differences. Variability in the time of rejection (13–20 days) may be caused by yet other non-*H-2* antigens. Since grafts of group 5 were also rejected later (20–40 days), segregation of other *H* antigens seems probable. White-cell agglutination reactions demonstrated that the antiserum would agglutinate peritoneal fluid cells. Thirty strains of mice were

Table 1.26.1. Antigenic Phenotype of (101 × RFM)F_2 and (C57BL × RFM)F_2 Offspring[a]

	$H\text{-}2^f$ Positive, *rho* Positive	$H\text{-}2^f$ Positive, *rho* Negative	$H\text{-}2^b$ or $H\text{-}2^k$ Positive, *rho* Positive	$H\text{-}2^b$ or $H\text{-}2^k$ Positive, *rho* Negative
Observed	28	11	10	3
Expected[b]	29.25	9.75	9.75	3.25

[a]The data from the two populations were pooled since the results were similar.

[b]Expected values for independent segregation of two factors in an F_2 population.

Table 1.26.2. Rejection Time (Days) of Tail Skin Grafts Exchanged Among 27 (101 × RFM)F_2 Mice[a]

Graft 1	Graft 2	Graft 3	Graft 4	Graft 5
Autograft	*H-2* alike, *rho* alike	*H-2* alike, *rho* different	*H-2* different, *rho* alike	*H-2* different, *rho* different
b	20–40	13–20	8–9	8–9

[a]All animals were tested for *H-2* and *ρ*. Each recipient received five grafts as described above. The period during which rejections were noted for each group are indicated. All grafts except the autografts were rejected by day 40.

[b]Five autografts were lost after 40 days.

tested by red-cell agglutination, and only one other strain (F/St) possesses this antigen on its red cells.

The tissue and strain distributions of the antigen suggest that it differs from other non-*H-2* antigens already described. The discovery of this and other non-*H-2* isoagglutinogens will help to expand our knowledge on the relationship of tissue transplantation and isoantigens. The antigen is tentatively called *rho*, ρ.

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2. Drosophila Cytology and Genetics

D. L. Lindsley, Jr.

Radiation Genetics (2.1–2.4)

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P. A. Roberts
W. E. Trout III
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Chromosomal Behavior and Biochemical Genetics (2.5, 2.6)

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2.1 THE MALE STERILITY OF X-AUTOSOME TRANSLOCATIONS

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Translocations between the X chromosome and either chromosome 2 or 3 are frequently sterile. Thus, in most experiments, where translocations are scored by progeny testing sons of irradiated fathers, a large proportion of T(X;A)'s are missed. Consequently, we have designed and used a method that allows us to recover translocations from the daughters of irradiated males, so as to eliminate any bias attributable to carrying the irradiated X in hemizygous condition.[1] The most recent tabulation of the results from the T(X;2)'s and the T(X;3)'s recovered from these experiments is presented in Table 2.1.1. The proportion that are lethal or sterile provides an indication of the bias existing in samples of translocations recovered from sons of irradiated males.

Cytological characterization and male fertility tests have been completed on the majority of the male-viable T(X;A)'s. The results for reciprocal translocations are plotted in Fig. 2.1.1. The fertile translocations seem either to have their X-chromosome breakpoint in the proximal heterochromatin or to have both breakpoints located toward the tips of chromosome arms. Thus it appears that, as long as the euchromatic portion

Table 2.1.1. Characterization of X-Ray-Induced Translocation Between the X Chromosome and Chromosomes 2 or 3

	Experiment	Lethal	Sterile	Fertile	Total
T(1;2)	1959	3	5	7	15
	1961	14	38	7	59
	Total	17	43	14	74
T(1;3)	1959	2	5	5	12
	1961	8	37	6	51
	Total	10	42	11	63
	Grand total	27	85	25	137

ORNL-DWG 65-3112

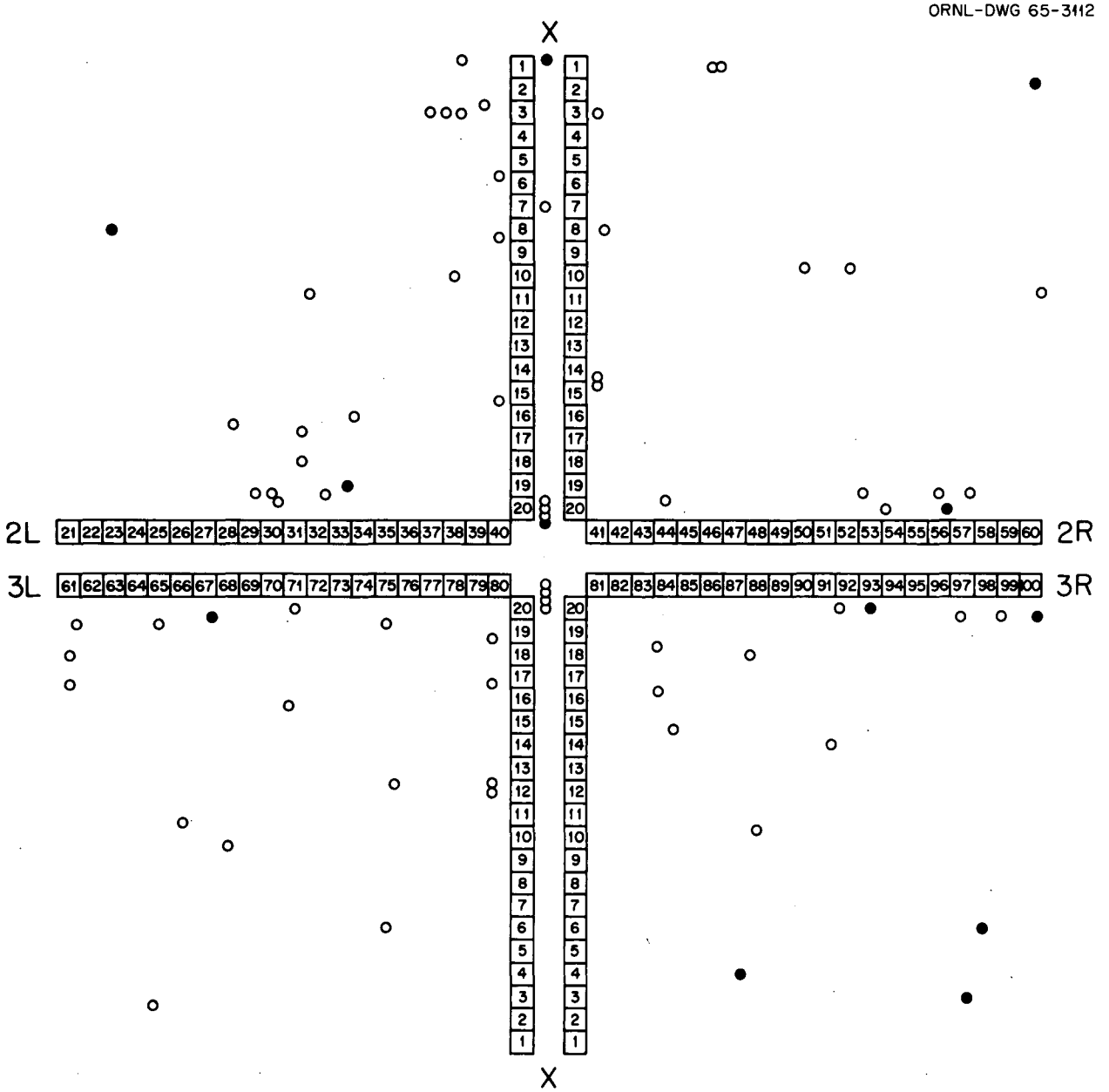

Fig. 2.1.1. Distribution of Breakpoints of the Viable Reciprocal Translocations Between the X Chromosome and Chromosomes 2 and 3. Solid circles represent male-fertile and open circles male-sterile T(X;A)'s. Points within the axes represent translocations with heterochromatic autosomal breakpoints in which the autosomal arm involved could not be ascertained.

of the X chromosome is largely intact and not capped by too large an autosomal fragment, an X-autosome translocation may be fertile. We feel that these data reflect the role of the X chromosome in spermatogenesis, but as yet have no adequate hypothesis.

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2.2 ANOTHER DIFFERENCE IN THE BEHAVIOR OF EU- AND HETEROCHROMATIN: CROSSING-OVER

P. A. Roberts

The recombination map length (0.04%) for the heterochromatin of the X chromosome of *Drosophila melanogaster* is far less than would be anticipated from its mitotic bulk (half the chromosome at metaphase) and considerably less than would be estimated from its polytene length (17 bands of 1024).[1] An exchange frequency comparable to the euchromatic average for the 66 map units of the X chromosome should result in a heterochromatic map length of at least 1.6% and possibly as high as 33%, depending on which cytological measurement more closely approximates the actual physical length of X heterochromatin. In order to decide whether this low frequency of crossing-over reflects a basic difference in the exchange behavior of eu- and heterochromatin rather than an overestimate of the physical length of X heterochromatin, use was made of the ability of autosomal inversions to increase crossing-over on the X chromosome, the interchromosomal effect on recombination.

The region between suppressor of forked (*su-f*, 66.0) and nonyellow Dp(sc^{V1} y^+) located on the short right arm of the X includes most of the X heterochromatin, while the carnation (*car*, 62.5) to *su-f* region includes the euchromatic-heterochromatic junction and adjacent euchromatin for comparison. Sisters of the genotype *y f car su-f/y f* · Dp(sc^{V1} y^+) with or without the autosomal inversions, SM1 and Ubx^{130}, were crossed to *y f car su-f*/Y males, and the F_1 scored for recombination.

It can be seen from Table 2.2.1 that the *car* – *su-f* region, which includes euchromatin, exhibits a pronounced increase in recombination in the presence of the autosomal inversions, while the region which is heterochromatic is refractory to the interchromosomal effect. The interchromosomal effect on recombination, which has hitherto been described as most pronounced in "heterochromatic regions," is more accurately described as pronounced in the proximal euchromatin but absent in heterochromatin.

Crossing-over apparently takes place in the early growth stages of the oocyte.[2] In spite of the generally unfavorable nature of the cytological material, heterochromatin is demonstrable in the oocyte nucleus throughout these early growth stages. Moreover, the nucleolus organizer is in X heterochromatin, and, when the nucleolus is first visible (stage 3) and in succeeding stages, it is seen to be associated with heterochromatin.[3] It has recently been demonstrated that condensed, pycnotic chromatin is relatively inactive in the synthesis of RNA compared with the more diffuse chromatin.[4] The present data make it possible to correlate another aspect of chromosome behavior with a particular cytological state and indicate a fundamental difference in the exchange behavior of eu- and heterochromatin owing, apparently, to the condensed state of heterochromatin at the time of exchange. It follows that the more diffuse chromatin condition is a prerequisite for the regular and frequent exchanges that occur during meiosis.

Table 2.2.1. Recombination in Eu- and Heterochromatin of the X Chromosome

Autosomal Inversions Present	Number of Flies Scored	Percent Recombination	
		Euchromatic *car – su-f*	Heterochromatic *su-f – y^+*
None	5005	3.54	0.04
SM1; Ubx^{130}	9278	9.07	0.04

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2.3 MOSAIC MALE STERILITY MUTATIONS IN DROSOPHILA

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J. L. Epler

In general, x-ray-induced sex-linked male sterility mutations in Drosophila are involved with X-autosome translocations. However, the monofunctional alkylating agent, ethyl methanesulfonate (EMS), induces a high frequency of mutations without

apparent chromosome breakage. Thus it is of interest to detect and characterize chemically induced sterility mutations and compare them with x-ray-induced effects. Since EMS-treated autosomes were available from mosaic lethal experiments,[1] this material was examined further for sterility mutations.

The genetic scheme employed detected male and/or female sterility mutations induced on chromosomes 2 and 3. In 47 treated genomes examined, one male sterile whole-body and seven male sterile mosaic mutations were found. In addition, three female sterile whole-body mutations were observed. Further characterization of these sterility mutants is in progress.

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2.4 CHEMICALLY INDUCED MOSAICISM IN DROSOPHILA

J. L. Epler

Studies of gonadal mosaicism in Drosophila have shown that a high frequency of mosaic recessive lethal mutations occurs after treatment of sperm with monofunctional alkylating agents. However, the mosaic frequency and accompanying complete lethal frequency are usually reported for only a single injected concentration and mutagenic efficiency. When the relative frequencies of gonadal mosaic and complete lethals induced by ethyl methanesulfonate (EMS) were assayed over a wide range of injected concentrations and mutagenic effectiveness, it was shown that, while the frequency of complete lethals is linear with injected dose, the frequency of mosaic lethals increases with dose at low concentrations and then declines at higher doses.

Preliminary results with coincidently occurring sex-linked lethal and visible mutants supported the hypothesis that the decline in mosaic frequency was explicable on the basis that many of the completes were, in fact, double gonadal mosaics (i.e., gonads composed of two separate lethal mutations and thus, in a sex-linked test, indistinguishable from a complete).

In order to test this hypothesis, a genetic scheme was developed to detect *autosomal* mosaic lethals. Each F-1 male gonad was scored as a nonlethal or as a complete, mosaic, or multiple mosaic lethal with respect to chromosomes 2 and 3. This was accomplished by establishing ten sublines representing each gonad. Each subline was checked for homozygous viability of the treated chromosomes and in combination with the other sublines from the same gonad.

The results obtained were (1) complete lethals, 22.2%; (2) mosaic lethals, 11.4%; and (3) multiple mosaics, 8.6%. Thus it was demonstrated that the decrease in *detectable* sex-linked mosaics might be attributed to the occurrence of multiple mosaic gonads.

2.5 REVISION OF *THE MUTANTS OF DROSOPHILA MELANOGASTER*

D. L. Lindsley, Jr. I. Lucille Norton
E. H. Grell Elizabeth S. Von Halle

Work continues on the revision of *The Mutants of Drosophila melanogaster*, which is a detailed catalog of all the genetic material, past and present, available for studies with the fruit fly. We undertook this task just two years ago, and we have been writing for approximately 15 months. To date, detailed descriptions have been prepared of nearly all mutations that have been described; this represents well over half the material that will go into the book. A good beginning has been made in writing descriptions of the chromosome aberrations. When the mutants and the chromosome aberrations are completed, the book will be approximately 90% finished. The remaining sections on special chromosomes, wild-type stocks, departures from diploidy, cytological markers, and nonchromosomally inherited factors will be very small.

In cooperation with C. E. Price of the Computer Services Division, we are preparing a master file on magnetic tape of all the material being described. From this file it will be possible to classify genetic alterations with respect to a number of different characteristics and prepare tables ordered on any characteristic or combination thereof. The initial stages of the machine program have been completed and seem to be in good working order. We anticipate that in the next semiannual report we will be able to state that our part of the task is finished.

2.6 CORRESPONDENCE BETWEEN THE TIMES OF DNA REPLICATION AND CROSSING-OVER IN THE OOCYTE OF *DROSOPHILA MELANOGASTER*

Rhoda F. Grell Ann C. Chandley

The time relationship between DNA replication and crossing-over in the oocyte of *Drosophila melanogaster* has been investigated.

Method. – The period of time that elapses between the exchange process and the mature oocyte has been measured. The measurement has been accomplished by the use of a 24-hr heat treatment at 35°C, which increases the frequency of crossing-over in the proximal regions of chromosome 2. Daily brooding of such heat-treated females permits one to determine the number of broods that intervene between treatment and the occurrence of a significant increase in exchange among her progeny. This interval represents the time between exchange and the mature oocyte. The assumption must be made that the heat acts directly on the exchange process.

The time that elapses between the last DNA replication and the mature oocyte has been independently estimated. When ^{3}H-thymidine is injected into females, it is incorporated into the DNA of the oocyte at the time of the last replication and remains undiluted by further replications. The progress of the labeled oocyte through successive stages until maturity has been followed cytologically by using daily samples of sectional ovaries from the injected females.

Results. – The time intervals between each of the two processes and the mature oocyte are given in Fig. 2.6.1. The first appearance of a mature labeled oocyte occurs on day 6, corresponding well with the first significant increase in exchange on day 6½. The majority of mature labeled oocytes appear on days 8–9 and correspond with the maximum increase in exchange on day 8½.

The labeling studies show that the incorporated ^{3}H-thymidine does not appear in stage 1 oocytes until approximately two days after injection, indicating that the last DNA replication of the germ cell takes place in the early 16-cell cyst located in the middle or anterior portions of the germarium.

Summary. – The results indicate that DNA replication and crossing-over are roughly coincident and suggest that both processes occur in the early 16-cell cyst in a stage which may correspond to the last premeiotic interphase.

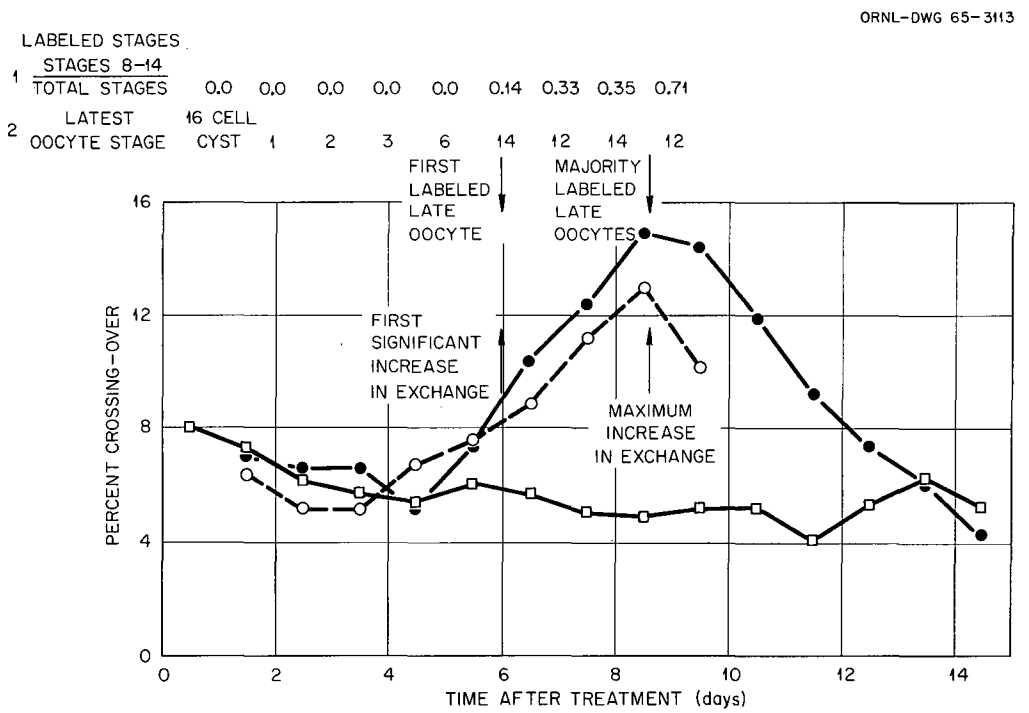

Fig. 2.6.1. The Correspondence Between the Times of DNA Replication and of Temperature-Induced Increase in Crossing-Over in the Oocyte of *Drosophila melanogaster*.

2.7 PRELIMINARY STUDY OF REVERSION IN THE NOTCH CISTRON OF DROSOPHILA

W. J. Welshons J. W. Day

The Notch cistron contains an array of seven recessive lethal and six recessive visible loci. The linear order of recessive lethals and visibles relative to each other is known. As a consequence, Notch may be considered as a well-mapped gene. By taking advantage of the recessive lethality inherent in the locus, it is possible to obtain data on very rare events occurring within the gene. Accordingly, we have commenced the study of reversion in the Notch cistron with the hope that a sensitive technique combined with relatively precise information on the marker distribution within the cistron will reveal the nature of the intragenic event.

In the course of mapping many recessive lethal Notches, we have observed that some regularly produce "reversions" to wild type in various heterozygous combinations, while others fail to do so. Three nondeficiency Notches producing reversions at an appreciable rate are N^{264-47}, N^{Co}, and N^{39}. Of these three, N^{39} was selected for the initial investigation.

When N^{39} was heterozygous with mutant sites fa^{no} or N^{40}, no reversions were found in a total of 147,000 tested chromosomes. However, in N^{39}/spl heterozygotes, six were obtained in a total of 205,000; and in an N^{39}/fa heterozygote, one was obtained in a total of 60,000. By contrast, deficiency Notches combined heterozygously with other mutant sites failed to produce revertants in over 345,000 tests.

With a known variation in technique it is possible to screen almost 5×10^5 chromosomes in a routine fashion. Experiments are now in progress to determine the reversion rate of N^{39} when it is heterozygous with other intragenic mutant sites. Similar experiments will be performed in the homozygous combination (N^{39}/N^{39}) and with N^{39} heterozygous for a deficiency Notch. Our intention is to determine the effect of the "other" chromosome on the reversion rate of N^{39}.

3. Cytochemistry and Cell Reproduction

R. C. von Borstel[a]	
Yeast Genetics (3.1) R. C. von Borstel[a] Martha J. Bond[a] T. R. Manney Margaret D. Schellinger K. C. Atwood[b]	**Cell Biochemistry (3.4, 3.5)** I. L. Cameron Betty M. Wysinger
Insect Cytology and Genetics (3.2, 3.3) J. G. Carlson[b] Diane J. Goins[a] Mary Esther Gaulden[a,b] Anna R. Whiting[a,b] P. W. Whiting[b]	[a]Dual assignments. [b]Consultant.

3.1 COMPARISON OF TRYPTOPHAN SYNTHETASE FROM YEAST AND *NEUROSPORA*

T. R. Manney Margaret D. Schellinger

Introduction. – As part of a project to determine the nature of supersuppressible mutants, we have been studying some properties of wild-type and mutant forms of tryptophan synthetase (TSase) from the yeast *Saccharomyces cerevisiae*. Genetic studies suggested that this enzyme might be similar to *Neurospora crassa* TSase. The latter enzyme has been purified in two different laboratories and has been rather well characterized. Accordingly, we have studied the yeast enzyme by comparing its properties with those of the *Neurospora* enzyme.

This report summarizes the similarities and differences we have found under three categories: (1) enzymatic properties, (2) immunlogical properties, and (3) physical properties.

Results. *Enzymatic Properties.* – The yeast enzyme catalyzes the same three principal reactions as does TSase from *Neurospora* and bacteria:

(1) indole-3-glycerol phosphate (InGP) + L-serine → L-tryptophan + glyceraldehyde phosphate

(2) indole (In) + L-serine → L-tryptophan

(3f) indole-3-glycerol phosphate (InGP) → indole (In) + glyceraldehyde phosphate

The relative rates for these reactions are compared with the relative rates reported for *Neurospora*[1] in the following table:

Reaction	*S. cerevisiae*	*N. crassa*
1	69	46
2	100	100
3f	5	9

In reactions (1) and (2) the amount of InGP or In removed always agrees with the amount of

tryptophan formed. In reaction (3f), however, there is always a significant and somewhat variable discrepancy in the amount of InGP removed and the amount of In formed. This discrepancy has been reported for *Neurospora*[2] but has not been explained.

The pH optimum for the yeast enzyme in reaction (2) is at 7.2, whereas in *Neurospora* it is reported to be at 7.8.[3]

Immunological Properties. – Using antisera against partially purified preparations from both organisms,[4] we have studied neutralization of reaction (2) activity and, to a limited extent, of reaction (3f). Both antisera appear to contain neutralizing antibodies against each of the enzymes; however, the heterologous reactions are much weaker than the homologous reactions (<10%). Both the amount of activity neutralized and the rate of neutralization are less. Reactions (2) and (3f) are neutralized by the same relative amount in the homologous yeast system.

Physical Properties. – The most significant difference in physical properties that we have found is the pH dependence of solubility in ammonium sulfate (about 30% saturated). The yeast enzyme has a sharp minimum at about pH 4.5, whereas the minimum for *Neurospora* is somewhat broader and occurs around pH 5.5.

On the other hand, yeast TSase is quite similar to *Neurospora* in its heat stability. Both are less heat labile in the presence of pyridoxal phosphate and serine. Further, a mixture of equal activities eluted from a Sephadex G-200 gel filtration column in a single peak indicates that they are very nearly the same size.

The yeast enzyme has been chromatographed on three grades of Sephadex (G-75, G-100, and G-200) and on diethylaminoethyl (DEAE) cellulose. In all cases, 70 to 100% of the starting activity has been recovered in a single peak, and no activity has been found in any other fractions.

Conclusions. – Aside from certain pH-dependent properties, yeast TSase appears to be similar to *Neurospora* TSase. The enzymatic activities are associated with a single protein that is not readily separated into subunits. This is in contrast to TSase from several bacteria, which is composed of two easily separated proteins.

In further studies similar techniques will be used to characterize the mutationally altered yeast TSase from a variety of mutants, suppressed mutants, and revertants.

References

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[2]M. D. Garrick and S. R. Suskind, *J. Mol. Biol.* **9**, 70–83 (1964).

[3]C. Yanofsky, *Methods Enzymol.* **2**, 233–38 (1955).

[4]The antiyeast serum was prepared by C. J. Wust; the anti-*Neurospora* serum was a gift from S. R. Suskind, Johns Hopkins University, Baltimore, Md.

3.2 VISIBLE MUTATION FREQUENCIES INDUCED IN MORMONIELLA OOCYTES BY X RADIATION

P. W. Whiting D. T. Ray[1]

Introduction. – The wasp Mormoniella has the conventional haploid-male, diploid-female mode of inheritance. Visible mutants for the entire genome are therefore easily detectable among haploid offspring. In this particular wasp it is possible to screen routinely for mutants among large numbers of offspring (10,000–100,000). Previous studies have been made of the mutation frequencies induced in oocytes exposed to x radiation between 1136 and 5680 r.[2,3] The present study is a summary of these data and some data collected from wasps irradiated with lower exposures (52.5–525 r). From the age of the females irradiated, it was expected that the results would represent mutations induced in oocytes in the first meiotic prophase stage of development.

Results and Discussion. – The accumulated results are shown in Fig. 3.2.1. The first four points represent mutants collected from around 150,000 wasps at each point, and higher exposures represent data from between 2700 and 17,000 wasps at each point. The accumulated spontaneous mutations in the controls are six mutants in 195,807 wasps examined.

The higher-dose data indicate that visible mutations are induced by more than one event, since the slope appears to be nearly 2. The lower-dose portion of the curve shows that, even when the data are corrected for spontaneous mutations (Δ), the slope is still less than 1. This

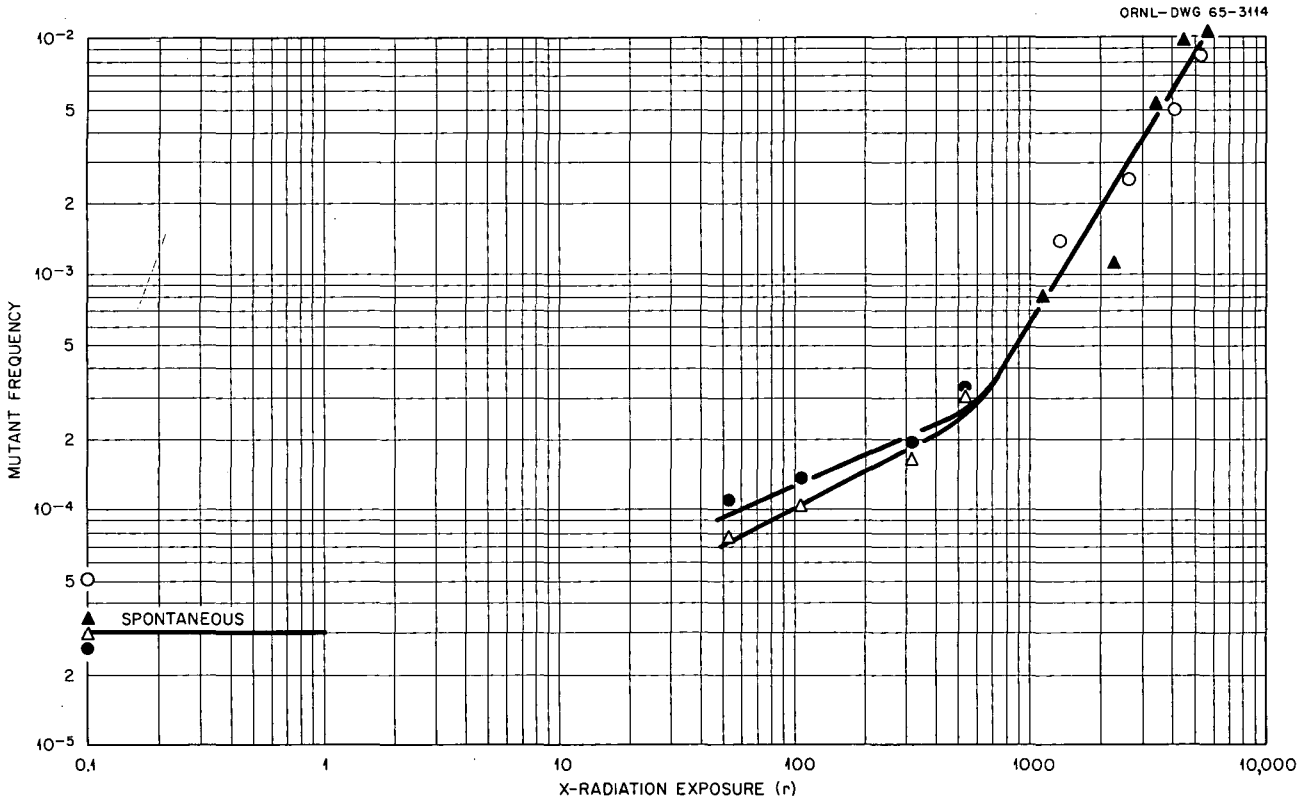

Fig. 3.2.1. Exposure-Mutation Curve for Visible Mutations Induced in Oocytes of Mormoniella. ● – unpublished data; O – Ray and Whiting, 1954; ▲ – Kayhart, 1956; Δ – total spontaneous, and unpublished data corrected for spontaneous mutants.

indicates that a small fraction of the irradiated oocytes was probably at a more sensitive stage of development when irradiated, and this slope represents a plateau that was reached before mutations were induced in the more resistant oocytes. Other interpretations are not excluded. For example, cumulative radiation damage to a mutation-repairing system can also explain the results.

References

[1]Department of Zoology, Howard University, Washington, D.C.

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3.3 THE "BLACK" EYE COLORS OF MORMONIELLA

P. W. Whiting

Introduction. – Eye-color mutants of this wasp may be grouped as "bright" or "black." All are recessive to wild-type brown. The "bright" colors are due to reduction or complete loss of "black" pigment, increase in bright pigment, and decrease or absence of all pigment. The resulting "bright" mutants have been called mahogany, dahlia, garnet, vermilion, scarlet, orange, peach, tinged, and oyster white. The "black" mutants are due to reduction or absence of all pigments except lavender and black.

Methods. – Crosses have been made of 13 different blacks with "bright" eye-color stocks designated dahlia-GF, vermilion-MK, scarlet-DR,

and orange-305. The wild phenotype F_1 females have been set unmated, and the colors and proportions of the F_2 males determined.

Results and Discussion. – The double recessives for seven of the blacks by dahlia-GF are black; for the remaining five they are lavender. The former are designated *eb* blacks because the first of these found was called ebony. Blacks giving lavender in combination with dahlia-GF are distinguished as *bk* blacks. Combinations of vermilion-MK or of scarlet-DR with the *eb* blacks are vermilion and scarlet, indistinguishable from the single mutant types. These two "bright"-color genes are genetically related, being different states or conditions of the same color factor. Combinations of the *bk* blacks with vermilion-MK are pale lavender; with scarlet-DR they are white. The *bk* black removing all of the bright red pigment leaves the small amount of "black" or lavender present in vermilion. Scarlet lacking "black" is changed to white.

Orange-305 is changed to white in combination either with *eb* black or with *bk* black.

Crosses were made between the different "whites" in order to determine homologies of the different blacks. The *eb* blacks all proved homologous because the F_1 females were always white. Crosses of the "*eb* whites" with the "*bk* whites" always gave colored F_1 females, indicating nonhomology as expected. By the same method there were found to be three different genetical loci among the five *bk* blacks.

Tests were made by breeding F_2 males from some of the colored F_1 females. Deviations from expected ratios may be explained by linkage of blacks giving excess of whites, by lowered viability of double recessives giving deficiency of whites, and, in a few cases, by induced chromosomal irregularities.

3.4 METABOLIC REGULATION OF THE DNA-SYNTHETIC PERIOD IN *TETRAHYMENA*

I. L. Cameron G. M. Padilla
Betty M. Wysinger

Introduction. – It has been shown in this laboratory that varying the amount of DNA in the macronucleus of *Tetrahymena pyriformis* did not change the duration of the DNA-synthetic period (S). It has also been demonstrated that all diploid somatic cells of mammals and birds have a constant S duration (in vivo or in vitro). Can S duration be altered by curtailing nutrient precursors, or does the cell "store up" substrate reserves for the DNA-synthetic reaction such that the nutrient environment has little or no influence on S duration?

Methods and Results. – To determine the effect of growth-medium composition on the doubling rate, length of macronuclear DNA-synthetic period (S), and its positioning within interphase, cells were cultured at 29°C in (1) a minimal synthetic medium, (2) minimal medium supplemented with 0.04% proteose peptone, and (3) an enriched medium (1.5% proteose peptone, 0.1% liver extract). During the exponential phase, cells were exposed to ^{3}H-thymidine, periodically sampled for autoradiography, and analyzed with respect to the various phases of the cell cycle. Analysis of labeling kinetics revealed the percent of time

Table 3.4.1. Analysis of the Cell Cycle of *Tetrahymena pyriformis* Grown Under Different Nutritional Conditions at 29°C

Strain	Medium	Generation Time[a] (min)	S_0 DNA Synthetic Index at Zero Time (%)	Percentage of Cycle Occupied by Each Period[b]			
				G_1	S	G_2	D
HSM	1.5% proteose peptone + 0.1% liver extract	163	30	18	33	38	11
HSM	Defined media + 0.04% proteose peptone	225	30	36	36	17.5	11
HSM	Defined media	870	32	48	39	10	3.4
GL-C	1.5% proteose peptone + 0.1% liver extract	135	52	5	47	35	13.3
GL-C	Defined media + 0.04% proteose peptone	214	56	32	43	14	11.2

[a]Generation time = mean culture doubling time.
[b]In reference to macronuclear S and cytokinesis.

spent in each period of the cell cycle as is shown in Table 3.4.1. These results show that the duration of the S period is proportionally constant; therefore, the absolute duration increases as the generation time increases. At the same time, the G_1 period is lengthened with a reduction in availability of nutritional substrates, suggesting that more time and preparation are needed to initiate DNA synthesis. At the same time, G_2 is shortened both in proportionate and absolute time. This suggests that substrates for the DNA-synthetic reaction are limiting in the less-rich medium. The amicronucleate strain, GL-C, shows the same nutritional dependent phenomena as strain HSM. However, the proportion of time spent in S period is greater than in the micronucleated strain HSM.

The effects of temperature and other conditions on the duration of the S period within the cell cycle are now being studied.

3.5 SPATIAL DISTRIBUTION OF DIVIDING AND DNA-SYNTHESIZING CELLS IN MOUSE EPITHELIUM

I. L. Cameron

Introduction. – Early cytologists observed that mitotic figures appeared in localized areas or groups in stratified squamous epithelium. Several recent cytologists have reanalyzed this same problem by using procedures which do not adequately take into account the spatial relationship of one dividing cell to the next. They conclude that mitotic figures are randomly distributed. This study was undertaken for the following reasons: (1) because the recently employed techniques would not *necessarily* detect the nonrandom spatial distribution of dividing cells, and (2) determinations of randomness or nonrandomness will support or eliminate possible regulatory mechanisms of cell proliferation operating in vivo.

Hypothesis. – If every cell in the germinal layer of an epithelium has the same probability of being in division or in DNA synthesis respectively, at any given time a frequency distribution of the number of nondividing or nonlabeled cells separating dividing or labeled cells in a cross section of that layer would approximate a geometric distribution.

Methods. *DNA Labeling Method.* – Eleven adult male Swiss albino mice were used. Four animals received an intraperitoneal injection of 10 μc of ^{3}H-thymidine (specific activity 0.36 curie/millimole) and were killed 1 hr after injection. Five animals received similar injections but were sacrificed at 6, 10, 16, 26, and 75 hr after injection. Two additional animals received a second injection 9 hr after the first one and were killed 1 hr after the second injection. Lower segments of the esophagus were removed and fixed in Bouin-Hollande's solution. Transverse histological sections were coated by dipping autoradiography (Kodak NTB2 emulsion), exposed for two weeks, and, after developing in Dektol, were stained with hematoxylin and eosin. Analysis of the spatial distribution of cells in DNA synthesis, that is, predivision cells, was carried out by recording the number of nonlabeled cells

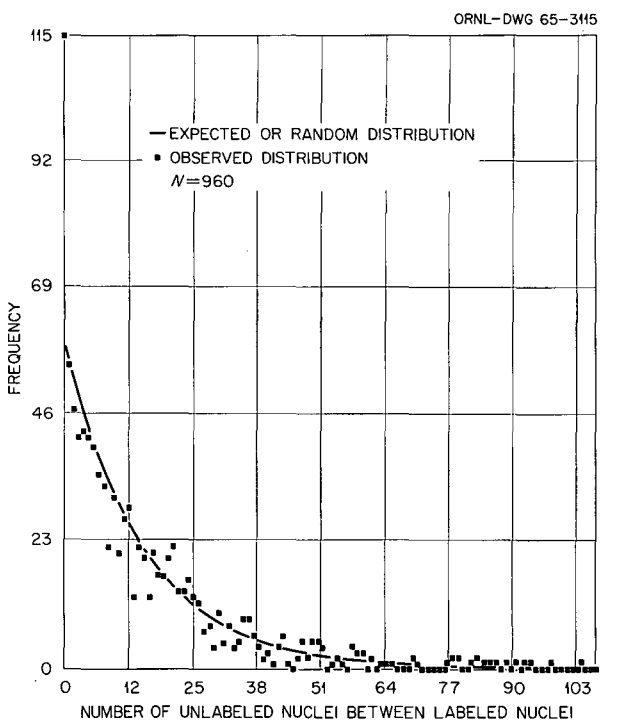

Fig. 3.5.1. Frequency-Distribution Histogram Showing the Spatial Relationship of DNA-Synthesizing Cells to One Another in the Germinal Layer of Esophageal Epithelium 1 hr After Injection of ^{3}H-Thymidine.

occurring between labeled cells along the length of the basal (germinal) layer of epithelium. As it is generally true that those cells in DNA synthesis will divide shortly after finishing their DNA replication, DNA synthesis therefore is an indication of the predivision state of cells.

Colchicine Method. – In this study four adult mice were injected subcutaneously with colchicine (1 mg/kg) 4 hr prior to sacrifice. Representative samples of the lower segments of the esophagus were fixed in Carnoy's fluid. Routine histological sections, both transverse and longitudinal, were prepared and stained with hematoxylin and eosin. The tissue was analyzed as described above.

Results. *Distribution of DNA-Synthesizing Cells Following ^{3}H-Thymidine Administration.* – Figure 3.5.1 is a frequency-distribution histogram showing the spatial relationship of DNA-synthesizing cells to one another in the germinal layer of esophageal epithelium 1 hr after injection of ^{3}H-thymidine. The observed distribution as seen in Figure 3.5.1 does not agree with the theoretical random (geometric) distribution because the zero group is much more frequent than expected. The results therefore demonstrate that cells in DNA synthesis are not randomly distributed along the basal layer of epithelium.

Table 3.5.1 shows a statistical analysis of goodness of fit in the zero class of the frequency distribution. There is very poor agreement. In a similar analysis the entire observed distribution did not fit the expected distribution. However, if the zero class was excluded from the analysis, the fit was good. The zero class represents two or more labeled cells which are adjacent to each other.

The doubly injected animals had no sections of germinal epithelium with 28 or more unlabeled cells in a row. The animals given a single injection of ^{3}H-thymidine at 1, 6, or 10 hr prior to killing have a considerable number of epithelial segments with 30 or more unlabeled cells in a row.

Conclusions. – The following conclusions can be stated:

1. Linearly disposed epithelial sheets such as the esophageal epithelium are well suited for the quantitative assessment of the spatial distribution of proliferating cells.

2. Dividing and DNA-synthesizing cells in the germinal layer of mouse esophageal epithelium are "almost" random in their spatial distribution. There is, however, an excess of labeled or dividing cells immediately adjacent to one another over what would be expected if the spatial distribution were random.

3. Two possible explanations for the excess of adjacent proliferating cells are suggested: (1) direct cellular interaction or local humoral influences and (2) phasing of adjacent cells which have a common line of descent.

Table 3.5.1. Tests of Fit for Zero Class

One Hour After ^{3}H-Thymidine Injection	Observed	Expected[a]	λ^2	P
Animal				
Black	28	15.8	9.51	<0.005
Red	24	13.3	8.56	<0.005
Blue	35	16.1	22.2	<0.0005
Green	28	14.4	12.9	<0.0005
Sum	115	59.6	53.1	<0.0005
Animal 4 hr After Colchicine Injection	25	12.2	13.3	<0.0005

[a] $f(x) = pq^x$. When $x = 0$, $f(x) = p$ and expected number = Np, where N = total number.

4. Cell Growth and Differentiation

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4.1 DISSOCIATION AND MOLECULAR WEIGHT OF β-LIPOVITELLIN

R. A. Wallace

The extent of β-lipovitellin (β-Lv) dissociation as a function of pH was determined between pH 5.8 and 10.5 by ultracentrifugation, and the least-squares fit to 13 points gave the linear regression equation

$$\alpha = 0.137\text{pH} - 0.65 ,$$

where α is the fraction of β-Lv present as monomer. Extrapolation of this equation indicates zero dissociation at pH 4.7 (the approximate isoelectric point) and complete dissociation at pH 12.0. The dissociation constants computed from the fitted curve were also plotted on a logarithmic scale against pH, the resulting slope (0.34) indicating a decrease in free energy of about 0.5 kcal mole^{-1} (pH unit increment)$^{-1}$.

Preliminary light-scattering studies on the kinetics of the monomer-dimer transformation (Fig. 4.1.1) indicate that on altering the pH the dissociation reaction approaches a new equilibrium in about 0.5 min, but the reassociation reaction requires about 50 min. The relatively long period required for reassociation to occur suggests that the separation of the monomer and dimer components approaches completion during short (15–30 min) periods of ultracentrifugation, and this supports the view that dissociation constants computed from their relative proportions in such hydrodynamic analyses have a reasonable validity.

Light-scattering measurements were also performed on β-Lv solutions over a range of concentrations at three pH values (Fig. 4.1.2). Dissociation is evidently independent of β-Lv concentration between 0.02 and at least 0.08%. The molecular weight of the monomer (M_m) was, therefore, computed from the weight-average molecular weight (M_w) obtained with 0.05% solutions of the monomer-dimer system using the relationship

$$M_m = M_w/(2 - \alpha) = R_{90}/Kc(2 - \alpha) ,$$

where Kc/R_{90} is experimentally determined. M_m was found to be 2.27×10^5, which is higher than anticipated from the previously reported value of 4.0×10^5 for the dimer (see Cook[1]). Part of this discrepancy arises from the application of a solvent-displacement correction to the dry weights used for determining β-Lv concentration, a measurement required for estimating refractive index increment, partial specific volume, and absorptivity. When this correction was applied to the

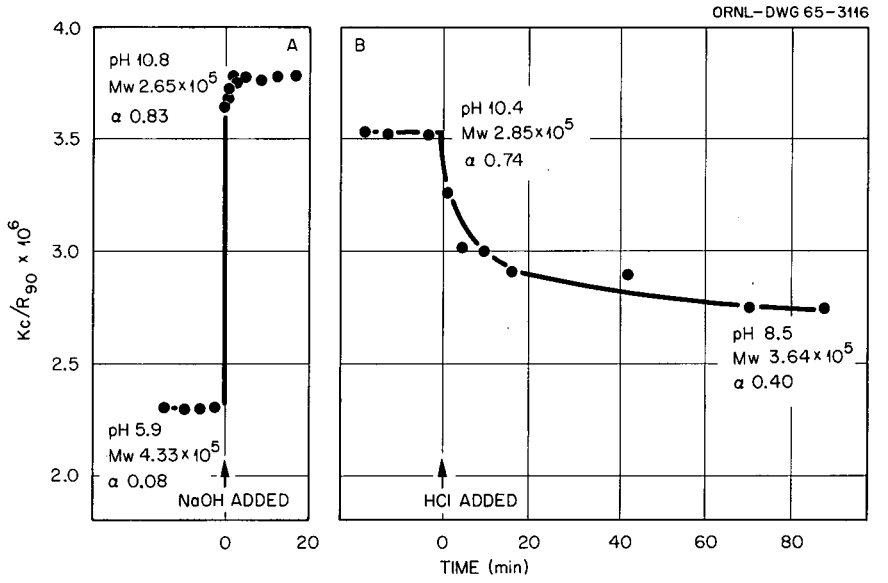

Fig. 4.1.1. Light-Scattering Properties of β-Lv in 0.5*M* NaCl Following Addition of NaOH (*A*) and HCl (*B*).

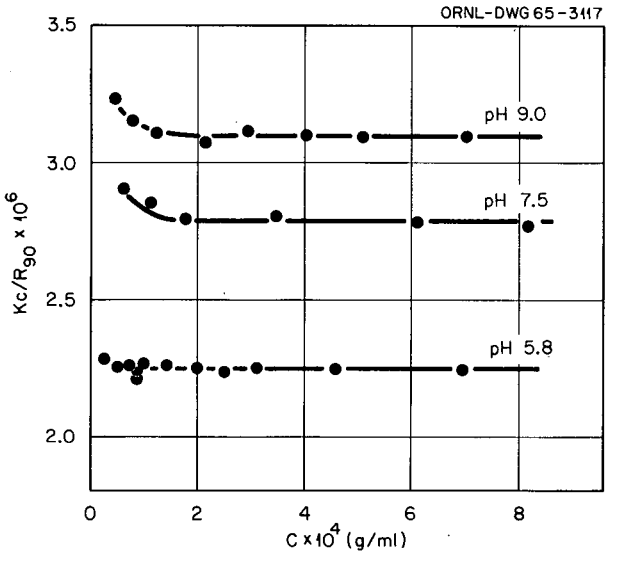

Fig. 4.1.2. Effect of pH and Concentration on Light-Scattering Properties of β-Lv in 0.5 *M* NaCl.

earlier results[1] obtained by hydrodynamic and light-scattering procedures, the molecular weight of dimer β-Lv was found to be 4.25×10^5 and 4.38×10^5 respectively. All these results are judged to fall within the experimental error applicable to an average value of 4.4×10^5 for the dimer.

This investigation concludes a series of studies on the physical and chemical properties of phosvitin and lipovitellin as isolated from mature vertebrate eggs. Further studies will now be undertaken to ascertain the manner in which these proteins are altered during transport to the egg, crystallization within the egg, and utilization during embryogenesis.

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4.2 FINE STRUCTURE OF LAMPBRUSH CHROMOSOMES

O. L. Miller, Jr.

Introduction. – Preliminary studies on the fine structure of lampbrush chromosomes isolated by a surface-film technique were reported previously.[1] In this method, surface-tension forces remove the ribonucleoprotein (RNP) matrix material from portions of the chromosomes, allowing observation of the deoxyribonucleoprotein axes of lateral loops.

In addition, individual units of the RNP matrices frequently are uncoiled or straightened in the surface film. Optimal viewing, however, is prevented by a background debris of nucleoplasm and sheared-off chromosomal matrix which is always present on preparations obtained by this technique. A microcentrifugation method for isolating these chromosomes subsequently has been developed. Using the centrifugation technique, the chromosomes can be separated from essentially all of the background material inherent in the surface-film technique.

Method. – For the microcentrifugation method, lampbrush chromosomes are manually isolated in the usual manner but in a chamber composed of a short length of narrow glass tubing glued to a cover slip. The dispersal of the nuclear contents is observed with an inverted-phase-contrast microscope. Next, an electron microscope grid is centered on the floor of a similar but larger chamber also filled with medium. A middle chamber, containing a funnel made from the highly tapered section of a Pasteur pipet, is inserted into the bottom chamber until the narrow end of the funnel is centered on the grid. After the chromosomes and nucleoplasm have spread sufficiently across the surface of the cover slip in the first chamber, the cover slip is inverted and placed on top of the middle chamber. The joined components of the centrifuge "tube" then are lightly centrifuged for 2 to 3 min. The relatively heavy weight of the chromosomes and nucleoli allows the clean separation of these organelles from the nucleoplasm. Further, the loop matrices can either be kept intact during the procedure or partially removed during or after centrifugation.

Following either isolation method, the chromosomes are precipitated with pH 4 buffer, then stained with 1 to 2% uranyl acetate at pH 5 for 15 to 60 min. The preparations then are dehydrated in ethanol and dried out of isopentane to minimize surface-tension distortion.

Results and Discussion. – Using either isolation procedure, observations with the electron microscope show the DNase-sensitive interchromomeric thread in main chromosome axes (contains two chromatids) to be 50 to 150 A in diameter, and the denuded axis of lateral loops (one chromatid) to measure 30 to 50 A in diameter. After partial trypsin digestion of centrifuged chromosomes, the thinner regions of main axes measure 30 to 50 A and the thinner parts of the exposed regions of loop axes are 20 to 30 A in diameter. These diameters are similar to those obtained by other methods for single double-helix DNA or DNP molecules and indicate that the chromatids of these chromosomes consist in width of single DNA or DNP molecules. These cytological measurements corroborate similar conclusions drawn by Gall from the kinetics of breakage of loops and main axes of lampbrush chromosomes by DNase.[2]

Two main types of RNP matrix material have been observed on lateral loops in centrifuged preparations. One type is found on a large majority of the loops and consists of clusters of ribosome-like particles interconnected by a network of thin fibers. The granules are 250 to 300 A in diameter and are spaced 1200 to 1500 A apart on the fibrous component. Estimates of the number of granules in single, large matrix units of this type indicate that these units would be 8 to 10 μ long if all the granules in one unit are attached to a single fiber.

A second type of matrix, which can be correlated with the "fuzzy" loops observed in the light microscope, is composed of well-defined fibrils ranging up to 10 μ in length in centrifuged preparations and up to 20 μ when stretched in surface-film preparations. When stretched in centrifuged preparations, the fibrils show granular units similar in diameter to those found loosely spaced in typical matrix units; but, in this case, the granules are closely packed on a thin axial fiber. Enzyme digestion results indicate that the matrix units are composed of an RNA fiber which, in one type, has a protein coat along its entire length and, in the other, has protein granules spaced periodically along its length.

Labeling with RNA and protein precursors shows immediate incorporation of label into both substances over the entire length of both typical and fuzzy-type loops (see Gall and Callan[3] for an exceptional-type loop). On the fine-structure level, the only morphological change occurring in individual matrix units from the thin to thick insertion ends of both types of loops is an increase in the length of the units. These observations suggest that the synthesis of RNA molecules is initiated repeatedly at the thin insertion end of each loop and continues around the entire length of a stationary loop axis, with each molecule continuously being "unzipped" from the loop axis as it is synthesized. They also indicate that the

protein component of loop matrices is synthesized on or incorporated onto the RNA molecules at the juncture of the loop axis and newly synthesized portion of each RNA molecule.

The large number of matrix units per loop suggests that a correspondingly high number of RNA polymerase molecules may be present on the loop axes. Estimates of the number of matrix units along loop axes suggest that the presumed polymerase molecules are spaced 1000 A or less along the entire length of each loop axis.

The longest matrix units so far observed (up to 20 μ in length) are longer than would be expected on the basis of lengths of RNA molecules estimated by other methods (length of polysomes, s values, necessary lengths for protein coding). This suggests that the RNP products of chromosomes may be subdivided into smaller units before functioning in protein synthesis, a process that is perhaps analogous to the subdivision of a large nucleolar RNA into the two sizes of ribosomal RNA reported by Perry.[4]

On the other hand, the longest matrix units so far observed are much shorter than loop axes which average 50 μ in length and range up to 200 μ in length. This suggests either that the entire length of the DNA in a loop axis may not be used for transcription or that the length of a loop axis does not consist entirely of DNA.

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4.3 A STABLE MITOCHONDRIAL DNA IN *TETRAHYMENA PYRIFORMIS*

G. E. Stone[1] O. L. Miller, Jr.

Introduction. – Evidence that mitochondria are semiautonomous organelles which contain both DNA and RNA was recently reviewed by Gibor and Granick.[2] More recently, Luck and Reich[3] reported RNA polymerase activity in a mitochondrial fraction from *Neurospora crassa* and the isolation of a low-molecular-weight DNA from the same fraction.

Incorporation of ^{3}H-thymidine into an acid-insoluble DNase-sensitive material in the cytoplasm of *T. pyriformis* was reported previously,[4] but no attempt has been made to further characterize this material. This report describes combined light- and electron-microscope autoradiographic experiments demonstrating that this DNase-sensitive cytoplasmic material is: (1) conserved in a manner which transmits approximately half of the labeled material to each daughter cell and (2) located within the mitochondria of the cell.

Results. – When logarithmically growing cultures of *T. pyriformis* are incubated in ^{3}H-thymidine (5–10 μc/ml; specific activity, 14.6 curies/millimole) for 30 to 60 min, autoradiographs of air-dried cells (3–4 weeks of exposure) show almost all of the incorporated radioactivity confined to the macro- or micronucleus. When incubation is prolonged, however, incorporated radioactivity is found in the cytoplasm. The cytoplasmic radioactivity is similar to the nuclear label in that it is removed by DNase digestion but not by buffer solution lacking DNase or by RNase. The rate of incorporation of ^{3}H-thymidine into the cytoplasm increases when the cells are deprived of essential amino acids; treated with actinomycin D or FUdR, or refed from a stationary phase condition. Some incorporation into the cytoplasm occurs during both the G_1 and G_2 stages of the cell cycle. However, an increase in the rate of incorporation occurs during the early part of the macronuclear S period, followed by an even higher peak of incorporation during the micronuclear S period.

To test the stability of this cytoplasmic material, the dilution of incorporated activity was followed through three complete cell cycles. Cells from stationary phase cultures were inoculated into fresh medium containing ^{3}H-thymidine and incubated until the cells began to divide. Dividing cells were selected, removed from the medium by using a braking pipet, and washed free of the isotope by repeated transfer through medium lacking isotope. After division was completed, one daughter cell of each pair was air dried on a slide, while the other daughter was allowed to continue through the next cell cycle. The process was continued through three divisions for each of 18 cell lines. The slides then were autoradiographed, and the number of silver grains over a standard area of cytoplasm was counted for each

cell. Grain counts for daughter cells of the second division were doubled, and those of the third division quadrupled. When the means of the three groups were compared, no significant difference was observed between division 1 and division 2 (Student's t test, $P = 0.30–0.20$) or between 1 and 3 ($P = 0.50–0.40$). These results indicate that the ^{3}H-thymidine-labeled cytoplasmic material undergoes little or no turnover during at least two complete cell cycles, and that approximately half of the labeled material in each cell is transmitted to each daughter cell during division.

To determine the sites of ^{3}H-thymidine incorporation in the cytoplasm, stationary phase cells were refed with fresh medium and then incubated for 4 hr with labeled precursor as in the dilution experiments. The cells were then processed for electron microscopy, and thin sections were covered with thin films of Ilford L4 emulsion for electron microscopy–autoradiography. A total of 428 silver grains were cataloged as to their location relative to mitochondrial profiles in sections of 21 cells; 73½% of the grains were located over mitochondria, 92% were located over or within 0.5 μ of mitochondria, and 97% were over or within 1.0 μ of mitochondria. The area of the cytoplasm covered by mitochondrial profiles was estimated from the distribution of a number of random points equal to the number of silver grains over the cytoplasm of each cell. Only 17.8% of the random points occurred over mitochondrial profiles, compared with 73.4% of the silver grains. Thus there were 12.7 times as many grains per unit area over mitochondrial profiles as grains per unit area over the rest of the cytoplasm. A frequency distribution plot of grains relative to the mitochondrial profiles further indicates that the radioactive DNase-sensitive material is located within the mitochondria and is not associated with the limiting mitochondrial membrane.

Discussion. – These results, when combined with the several previous reports of DNA in mitochondria in a variety of organisms, strongly suggest that the mitochondria of all cells contain a DNA-like material. Our results also indicate that this material is stable and is transmitted from one cell generation to the next in a manner which is analogous, at least superficially, to the distribution of nuclear DNA.

The occurrence of a stable DNA in mitochondria raises questions concerning the location of the necessary enzyme systems and associated nucleoside and nucleotide pools known to be involved in DNA synthesis. The association (although incomplete) of mitochondrial DNA synthesis with nuclear DNA synthesis suggests that some connection between the two may exist. Reports of actual fusing of nuclear and mitochondrial membranes point to the possibility that mitochondria may be directly dependent on the nucleus for at least part of the materials involved in DNA synthesis. On the other hand, we have never observed a direct structural association between either labeled or unlabeled mitochondria and nuclei in electron micrographs of hundreds of *T. pyriformis*. Although highly transitory connections cannot be ruled out, it seems unlikely that mitochondria are directly dependent on the nucleus for materials involved in the synthesis of mitochondrial DNA.

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4.4 DNA SYNTHESIS DURING EMBRYONIC INDUCTION

S. K. Brahma Tuneo Yamada

As a part of our project on cell biological analysis of amphibian embryonic induction, the pattern of DNA synthesis in amphibian ectoderm cells as affected by induction was studied in the following in vitro system. A piece of the gastrula ectoderm was halved. One of the halves was fused with a piece of dorsal mesoderm and cultured in vitro. The ectoderm was induced to form neural tissue under this condition. As a control, the other half of the ectoderm was cultured without addition of dorsal mesoderm. The control ectoderm differentiated only epidermal cells. At different time intervals from the beginning of the culture, explants were exposed to ^{3}H-thymidine, and uptake of isotope in the ectoderm nuclei was studied by autoradiography. From earlier embryological experiments we did not expect a pronounced change

in the pattern of DNA synthesis in the ectodermal cells as a result of inductive influence from the dorsal mesoderm.

Method. – Explants were prepared from the early gastrulas of *Triturus pyrrhogaster* in the same manner as has been described in our previous report. The experimental explant with mesoderm and the control explant without mesoderm were cultured for 1, 3, 6, 22, and 48 hr in Holtfreter solution at 18°C, exposed to 2 μc/ml of ^{3}H-thymidine in Holtfreter solution for 3 hr, and fixed in acetic acid–alcohol. Paraplast sections 5 μ thick were treated with cold trichloroacetic acid (TCA) and covered with Kodak NTB-3 liquid emulsion. Some of the slides were incubated with DNase.

Results and Discussion. – The results summarized in Table 4.4.1 indicate that the series cultured for 1, 3, and 22 hr have the common feature that a slightly higher proportion of cells are labeled in the experimental explant in a majority of pairs tested. The 6-hr series show, however, a quite different tendency. Namely, all pairs indicate a significantly lower labeling frequency in the experimental than in the control. On the other hand, in the 48-hr series all pairs have a higher frequency of labeling in the experimental than in the control counterpart. Now the period around 6 hr after the beginning of the experiment has been shown to be the stage in which experimental ectoderm cells produce significantly more RNA than the control cells.[1] A suggested possibility is that around this stage of embryonic induction the ectoderm cells are synthesizing RNA under the influence of inducing factors coming from the dorsal mesoderm, and that this RNA synthesis delays replication of DNA. The 48-hr series correspond to the time of initiation of neural differentiation in the experimental ectoderm. The high frequency of labeled cells may indicate cell multiplication connected with neural differentiation. On the other hand, the control ectoderm, which is at this stage beginning differentiation of epidermal cells, indicates a decline in labeling frequency compared with the preceding stages.

Table 4.4.1. Frequency of Cells Labeled with ^{3}H-Thymidine

Hours	Experiment	Proportion of Labeled Cells	
		Experimental	Control
1	A	0.4025[a]	0.3304
	B	0.5714[a]	0.4043
	C	0.3596[a]	0.2865
	D	0.4207	0.5852[a]
3	A	0.3510[a]	0.2605
	B	0.6140[a]	0.3425
	C	0.1603	0.3824[a]
	D	0.2996[b]	0.2797
6	A	0.2140	0.6074[a]
	B	0.5515	0.7340[a]
	C	0.4195	0.6826[a]
22	A	0.6624[a]	0.5717
	B	0.5850[a]	0.4733
	C	0.5278[b]	0.5607[a]
	D	0.6617[a]	0.4590
48	A	0.3878[a]	0.1654
	B	0.6165[a]	0.2219
	C	0.4480[a]	0.0936
	D	0.6229[a]	0.4499

[a]Larger of the two proportions.
[b]Difference not significant at the 5% level.

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4.5 PATTERN OF PROTEIN SYNTHESIS DURING EMBRYONIC INDUCTION

S. K. Brahma Tuneo Yamada

The pattern of protein synthesis in the amphibian ectodermal cells during the process of neuralization in vitro was compared with that in the control ectodermal cells by means of autoradiography. The explants were prepared from early gastrulas of *Triturus pyrrhogaster* in a manner similar to that described elsewhere in this report.[1] The pair of experimental and control explants was cultured at 18°C for different intervals of time (1, 3, 6, 22, and 48 hr) and then incubated with ^{3}H-leucine (2 μc/ml) for another 3 hr at 18°C before fixation in acetic acid–alcohol.

Paraplast sections were cut 5 μ thick, and the slides were treated with cold TCA before dipping them into Kodak NTB-3 liquid emulsion.

Results and Discussion. – Silver grains were counted per unit area of the nucleus and cytoplasm separately, and the data were compared between the pair of experimental and control ectodermal cells derived from the same ectoderm (Table 4.5.1). Irrespective, whether the experimental or the control, the nucleus always showed the higher counts. In both experimental and control explants of the 3-hr series, the cytoplasmic incorporation was very low or almost negligible. The average ratio of grain count per unit area of the nucleus between the experimental and the control showed a maximum at 3 hr (Fig. 4.5.1). It has been reported that at 6 hr, RNA synthesis in the nuclei of the experimental explants was enhanced and the DNA synthesis was suppressed.[1,2] In the present result we find that the relative increase of protein synthesis in the nucleus preceded the time of enhancement of RNA synthesis. The present result seems to suggest the importance of nuclear protein synthesis in the ectodermal cells reacting to the inductive stimulus from the dorsal mesoderm.

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Table 4.5.1. Grain Counts per Unit Area of Nucleus and Cytoplasm

Hours	Experimental Nucleus	Control Nucleus	Experimental Cytoplasm	Control Cytoplasm
1	17.71 ± 0.17	22.71 ± 0.24	4.92 ± 0.07	7.13 ± 0.09
	10.58 ± 0.08	16.25 ± 0.17[a]	3.63 ± 0.05	5.08 ± 0.05
	21.04 ± 0.27	24.00 ± 0.23	12.79 ± 0.17	12.33 ± 0.09
	17.96 ± 0.18	16.67 ± 0.12	6.50 ± 0.07	5.71 ± 0.07
3	13.58 ± 0.22	11.17 ± 0.15	0.38 ± 0.41	0.38 ± 0.44
	17.46 ± 0.23[a]	8.88 ± 0.11	0.88 ± 0.02[a]	0.25 ± 0.02
	15.54 ± 0.17[a]	7.46 ± 0.65	0.29 ± 0.47	0.33 ± 0.59
	21.04 ± 0.25[a]	14.75 ± 0.12	0.42 ± 0.002	0.21 ± 0.04
6	16.00 ± 0.14[a]	10.13 ± 0.09	6.54 ± 0.05[a]	5.04 ± 0.04
	10.42 ± 0.08	15.54 ± 0.09[a]	4.21 ± 0.08	6.29 ± 0.03[a]
	23.67 ± 0.26	25.04 ± 0.19	9.33 ± 0.12	14.83 ± 0.14[a]
	25.79 ± 0.29	26.88 ± 0.21	10.25 ± 0.12	11.83 ± 0.14
22	26.75 ± 0.22[a]	19.75 ± 0.15	10.67 ± 0.13	10.21 ± 0.95
	20.71 ± 0.11[a]	16.54 ± 0.92	8.54 ± 0.11	8.00 ± 0.72
	19.42 ± 0.17	19.88 ± 0.12	6.83 ± 0.71	6.42 ± 0.44
	27.63 ± 0.26[a]	19.54 ± 0.15	13.04 ± 0.18	11.29 ± 0.10
48	27.29 ± 0.26[a]	14.25 ± 0.95	12.71 ± 0.12[a]	5.50 ± 0.58
	49.67 ± 1.74[a]	21.42 ± 0.14	30.54 ± 0.26[a]	10.38 ± 0.76
	29.08 ± 0.20[a]	16.67 ± 0.95	10.58 ± 0.11	9.17 ± 0.80
	29.96 ± 0.13[a]	21.33 ± 0.15	12.92 ± 0.23	10.79 ± 0.98

[a]Indicates difference is significant at 95% confidence limits.

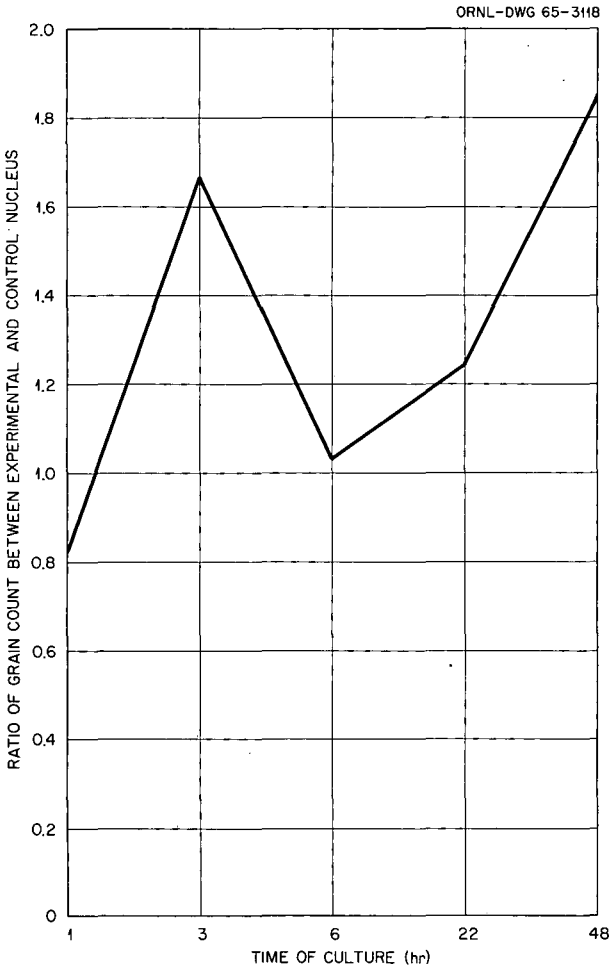

Fig. 4.5.1. Ratio of Grain Counts per Unit Nuclear Area Between Experimental and Control Series.

4.6 ENHANCEMENT OF RNA SYNTHESIS IN THE CELLS DURING WOLFFIAN LENS REGENERATION

Tuneo Yamada

Cell-labeling experiments with ^{3}H-thymidine and electron microscopic studies of Wolffian lens regeneration indicate that the cells of the dorsal iris go through a series of developmental processes during the regenerative tissue transformation. Latent, depigmenting, cell-division, and fiber-differentiating phases can be distinguished. In the present and subsequent experiments presented in this report, quantitative autoradiographic data are summarized, which allow us to follow the pattern of cellular RNA synthesis as it changes from one phase to the other during the whole course of cellular transformation in *Triturus viridescens*. The work described here specifically concerns the latent and depigmentation phases, in which methacrylate sectioning was required for autoradiography, due to the presence of pigment granules.

Methods. – Unless otherwise indicated, adults of *Triturus viridescens* were used as the material. Incorporation of ^{3}H-cytidine into the acid-insoluble fraction of autoradiographic sections was used as a measure for RNA synthesis. Since the precursor is incorporated not only into RNA but also into DNA in a part of the present experiments, a careful study was made with RNase to assess the extent of the RNase-insensitive fraction of radioactivity for each cell population selected for grain counting. Ten microcuries of ^{3}H-cytidine per gram of body weight was injected into the body cavity of animals at different time intervals after unilateral lens removal. Methacrylate sections of 0.5 μ were prepared through irises of the left operated and right unoperated eyes. A part of the serial sections was treated with RNase (37°C) followed by cold TCA, while the other part was treated with buffer without the enzyme (37°C) followed by cold TCA before covering with liquid emulsion. To avoid variation in the level of radioactivity from animal to animal, comparison of grain counts was only made between irises of the same animal.

Results and Discussion. *Enhancement of Nuclear Synthetic Activity in the Dorsal Iris After Lens Removal.* – The data presented in Table 4.6.1 indicate that incorporation of isotopes into RNA per unit nuclear area is enhanced in the dorsal iris in all phases of regeneration studied here. The ratio between grain counts per unit nuclear area of the left dorsal marginal iris and right dorsal marginal iris is higher than 3 and tends to increase with the progress of regeneration. This increase in the ratio in the later phase is significant even when data are corrected for the RNase-insensitive fraction, which increases in the later phase. Enhancement of nuclear RNA synthesis in the iris cells after lens removal has been already suggested by our earlier autoradiographic study,[1] which differs from the present study in adopting grain counts *per nuclear section* not corrected for the RNase-insensitive fraction as the basis of comparison. Although the earlier

Table 4.6.1. Autoradiographic Data on Incorporation of ^{3}H-Cytidine into Regenerate and Control Tissues

Experiment	Regeneration Stage	Ratios Between Grain Counts per Unit Nuclear Area[a] of			
		Experimental Dorsal and Control Dorsal	Experimental Ventral and Control Ventral	Experimental Dorsal and Experimental Ventral	Control Dorsal and Control Ventral
a	I	3.36	2.78	1.68	1.39
b	I	3.22	2.58	1.69	1.54
c	I	3.75	2.59	2.56	1.60
d	II_A	4.47	2.72	3.90	2.53
e	II_B	3.91	3.89	0.97	0.96
f	II_B	5.00			
g	II_B	5.20			
h	II_B	5.46	4.33	1.82	1.44
i	II_B			2.66	
j	II_B	5.85			
k	II_B	6.86	4.24	1.67	1.03
l	III_A	5.84	2.07	2.81	1.00
m	III_A	9.34	6.69	2.05	1.61

[a]All grain counts were corrected for the RNase-insensitive fraction.

method was admittedly less accurate than the present one, the general conclusion reached by the earlier study is now confirmed by the present data. Since, during the phase covered by both studies, the size of nuclei gradually increases, the ratio of grain counts reported in the earlier study should be expected to be higher than in the present one. This is generally the case.

Enhancement of Nuclear Synthetic Activity in the Ventral Iris After Lens Removal. – The ratio between grain counts per unit nuclear area of left ventral and right ventral irises reported in Table 4.6.1 suggests an enhancement of RNA synthetic activity also in the ventral iris of the operated eye. Except in one case, the ratio is less than that for the dorsal irises. This enhancement is noteworthy in the sense that the ventral part of the iris does not directly take part in the formation of the new lens. It has been reported that similar enhancement occurs also for incorporation of leucine and thymidine.[2] Unlike in the dorsal area, this enhancement of macromolecular synthesis is reduced in the subsequent stages of regeneration.

Comparison of the RNA Synthetic Activity Between Dorsal and Ventral Halves of the Normal Iris. – In the majority of cases studied, the average nuclear grain counts were significantly higher in the dorsal than in the ventral part of the nonoperated eye. It is possible that an inherent difference is present between two areas in RNA synthetic activity. A higher uptake of ^{34}P in the normal dorsal iris compared with the normal ventral iris has been reported.[3]

Experiments with Larval Tissues with Inhibited Pigment Formation. – It is possible to partially suppress pigmentation of iris by treating embryos of *Triturus* with phenylthiourea, without affecting lens regeneration capacity.[4] Under favorable conditions, the iris tissue from the larvae thus treated

can be studied in paraffin sections with conventional autoradiography. Attempts were made to utilize this situation for the present purpose of comparing RNA synthetic activity of the iris cells engaged or not engaged in lens regeneration. The data obtained with phenylthiourea-treated larval *Triturus viridescens* are in conformity with the data presented above. The ratios between the average grain counts per unit tissue area of the left experimental dorsal iris and the right control dorsal iris at regeneration stage II were 2.10, 2.49, and 3.32. That the values are less than the corresponding ones for the adults may be attributed to a higher normal synthetic level of the larval tissues.

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4.7 PROGRESSIVE CHANGES IN THE PATTERN OF RNA SYNTHESIS DURING DIFFERENT PHASES OF CELLULAR TRANSFORMATION IN WOLFFIAN LENS REGENERATION

Tuneo Yamada

To follow the changes in the pattern of RNA synthesis after the cells have entered the multiplication phase during Wolffian lens regeneration in the adults of *Triturus viridescens,* a number of autoradiographic series were carried out, using conventional paraffin sections as well as methacrylate sections. Special attention was given to the intracellular distribution of radioactivity, which is the function of the time interval between injection of isotopes and fixation of tissues as well as of the phase of cellular transformation. Ten microcuries of ^{3}H-cytidine per gram of body weight was injected into the body cavity of animals at various stages of regeneration, and regenerates were fixed at different time intervals after injection.

Comparison of the Level of RNA Synthesis Between Cells in the Multiplication Phase and the Fiber-Differentiating Phase. – In a series of autoradiographs, grain counts per unit nuclear area were compared for cells in the multiplication phase and those in the early fiber-differentiating phase. In counting the latter type of cells, only the superficially lying cells were used, in order to exclude the possibility that low diffusibility of isotopes through the fiber area affects the outcome of counting. The data summarized in Table 4.7.1 indicate that in the lens vesicle stage the cells in the multiplication phase show significantly higher grain counts than those in the early phase of fiber differentiation. The difference is consistently significant even if the RNase-insensitive fraction of grains, which is only present in the multiplication phase, is subtracted from the counts. Further data obtained with the growing lens are in good agreement with the above results. To check the possibility that observed difference in grain counts is caused by a difference in availability of isotopes to the different layers of regenerate, in which each type of cell is localized, a series of in vitro labeling experiments was done in which the regenerates and normal lenses were kept in a culture medium with ^{3}H-cytidine, under constant moderate agitation. The autoradiographic study of those tissues again showed a higher incorporation into the cells in multiplication than into those in fiber differentiation.

Change in the Intracellular Distribution of Radioactivity. – A series of autoradiographs was prepared at different time intervals after isotope injection into the lentectomized animals at the same regeneration stage. First, the data for the cells in the multiplication phase present in the external layer of lens vesicle (stages IV–VI) will be reported. In the 45-min and 3-hr series, the grains were localized mainly over the nuclei. Later, cytoplasmic grains increased in number. The percentage of cytoplasmic grains to total grains increased from 3 to 6 hr (Fig. 4.7.1). Closely similar data were obtained with the cells in the internal wall of the lens vesicle; these cells have entered the fiber-differentiation phase. Similar results were also obtained with the cells in multiplication in the lens epithelium of the growing regenerated lens (stages X–XI), as well

Table 4.7.1. Comparison of Incorporation of ^{3}H-Cytidine in Different Layers of Lens Regenerate

Time Interval Between Injection and Fixation	Grain Count per Unit Nuclear Area[a] (average of 100 counts)		Ratio, External/Internal
	External Layer	Internal Layer	
45 min	4.97	1.85	2.6
45 min	1.75	0.85	2.0
3 hr	9.52	3.99	2.3
3 hr	6.42	2.16	2.9
6 hr	5.13	2.38	2.1
6 hr	7.33	2.61	2.8

[a]Values corrected for RNase-insensitive grain counts.

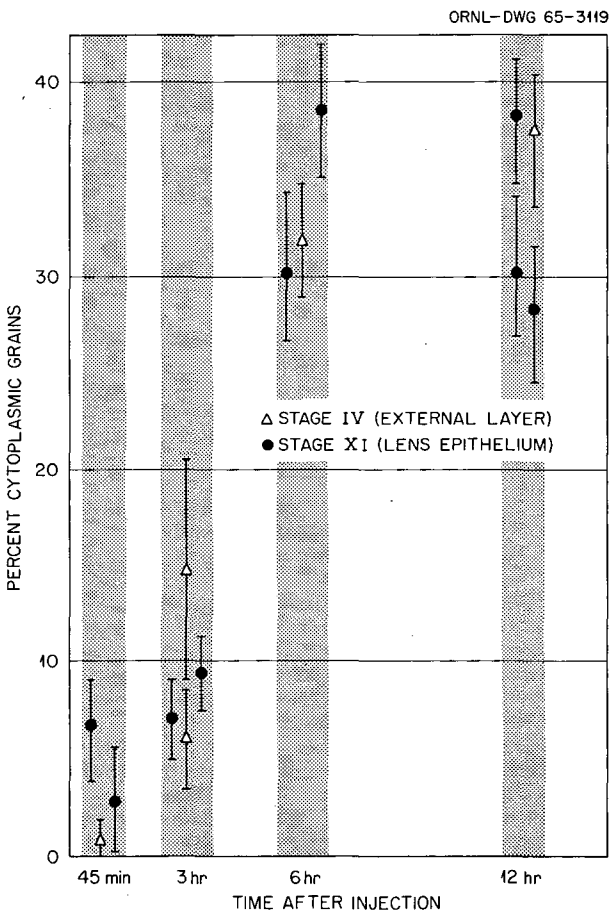

Fig. 4.7.1. Percentage of Cytoplasmic Grains at Different Time Intervals After Injection of Isotope.

as with the fiber cells in the same lens. On the other hand, the cells in the early depigmentation phase behaved quite differently. The percentage of cytoplasmic grains remained low throughout 45 min to 12 hr from isotope injection. All the above data were checked with RNase in each case. In the cells in multiplication as well as in fiber differentiation, the RNase-insensitive fraction of radioactivity was not above the background level until 3 hr after injection. Later, the fraction became significant and increased. At this phase the percentage of the RNase-insensitive fraction varied from 12 to 19% of total nuclear grains. In the cells of the late depigmentation phase, similar data were obtained. On the other hand, the RNase-insensitive fraction remained insignificant throughout those time intervals in the fiber differentiation, early depigmentation, and latent phases, as well as in the normal iris.

The data presented here, together with the data of the preceding paper,[1] indicate that the cells involved in regenerative tissue transformation go through a sequential change in the pattern of RNA synthesis. Soon after the lens removal, nuclear RNA synthesis in the iris cells is activated. The level of nuclear RNA synthesis gradually increases during the depigmentation phase. Until the later part of depigmentation, the shift of radioactivity into the cytoplasm remains minimum. After the cells enter active multiplication, from

the later part of the depigmentation phase on, this shift becomes noticeable. Probably this shift is mainly due to formation of ribosomes, which were shown to occur during the multiplication phase by electron microscopic data. When the cells enter the fiber-differentiation phase after cessation of DNA synthesis, the level of nuclear RNA decreases. However, the shift of radioactivity into the cytoplasm continues. Since in this phase an active production of ribosomes is absent, according to electron microscopic data, this cytoplasmic radioactivity should be accounted for by nonribosomal RNA.

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4.8 TEMPORAL SEQUENCE OF DNA SYNTHESIS AND TISSUE SPECIFIC-PROTEIN SYNTHESIS IN THE NEWT LENS REGENERATE STUDIED BY IMMUNOFLUORESCENT AUTORADIOGRAPHY

Chinami Takata

Introduction. – Previous studies have shown that during Wolffian lens regeneration, α- and/or β-crystallins first appear in the prospective primary lens fiber cells at the lens vesicle stage (stage IV);[1] and later, γ-crystallins become detectable in those cells which begin to elongate (stage V).[2] On the other hand, according to an autoradiographic study with ^{3}H-thymidine, when the lens vesicle is formed (stages IV–V), the prospective lens fiber cells, which are localized in its internal wall, stop synthesizing DNA.[3] These results suggest that lens specific-protein synthesis begins after the last DNA synthesis, and that the time interval between the last S phase and the beginning of protein synthesis differs according to crystallins.

The present experiment has been carried out to obtain information about the temporal relationship between DNA synthesis and specific protein synthesis in the lens regenerating system. For this purpose a technique was worked out that allows observation of autoradiographic grains and immunofluorescence in the same tissue section.

Materials and Methods. – The newts (*Triturus viridescens*) at 11, 15, and 18 days after lens removal were injected intraperitoneally with ^{3}H-thymidine (1 μc per gram of animal weight), and, at the end of a labeling period of 30 min, the excess of unlabeled thymidine was injected into each animal. At intervals of 30 min, 1, 3, 6, and 12 hr, and 1, 2, 3, 4, 5, and 6 days after the injection of thymidine, the regenerates were fixed with cold 95% ethanol, embedded in paraffin, and sectioned at 3 μ. After the slides were stained with the lens antiserum or the γ-crystallin antiserum conjugated with isothiocyanate, they were autoradiographed and exposed for 12 to 15 days. According to our immunochemical data, the lens antiserum used here is specific for α-, β-, and γ-crystallins of the newt, and the difference between the two antisera used here can be accounted for by α- and β-crystallins.

Results and Discussion. – Labeled cells appeared in the regenerates 20 min after the injection of ^{3}H-thymidine. The average grains per cell reached a maximum at 2 to 3 hr, followed by a decrease to about half the maximum value within 12 hr after injection. Thirty minutes after the injection of ^{3}H-thymidine, the labeled cells were distributed over the whole area at stages III–IV, in the prospective epithelial and equatorial area at stages V–VI, and only in the epithelium at stages VII–VIII. None of those labeled cells indicated immunofluorescence for lens specific antigens or for γ-crystallins. Twelve hours after injection, the lens specific antigens were detected for the first time in a few labeled cells in their cytoplasm in the fiber area. But immunofluorescence for γ-crystallins could not be detected in any of the labeled cells at this time interval. The presence of γ-crystallins was first recognized in labeled cells about two days after injection, when those cells were localized in the lens fiber area. The relative change in the distribution patterns of silver grains and immunofluorescence during the time course studied here indicates that those cells showing both immunofluorescence and silver grains were labeled during their last S phase. The above data of grain counts indicate that those labeled cells went through cell division within 12 hr after injection.

Taken together, the present results suggest that α- and/or β-crystallins begin to be detected from 12 hr after the last S phase and shortly after the last cell division, whereas γ-crystallins begin to

be detected from 2 days after the last S phase and from more than 1 day after the last cell division.

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4.9 THE NUCLEOLUS AND RNA SYNTHESIS DURING THE DEVELOPMENT OF MARINE INVERTEBRATES

Shuichi Karasaki

Introduction. – According to recent biochemical analysis[1,2] of sea urchin embryogenesis, the synthesis of the three types of RNA is initiated at different periods of development. During cleavage, DNA-like RNA and large quantities of soluble RNA are synthesized. Ribosomal RNA begins to appear after the gastrula stage, along with the first appearance of definitive nucleoli. On the other hand, in some mollusca, prominent nucleoli are present already at the cleavage stage. In the present electron microscopic and autoradiographic studies, the sites of nuclear RNA synthesis during embryogenesis of sea urchin (*Arbacia punctulata)* were examined at the ultrastructural level. Special attention was paid to the relation of the pattern of RNA synthesis to formation of nucleoli. Molluscan embryos, *Spisula solidissima* (surf clam), in which large nucleoli are already present at cleavage stage,[3] were similarly examined.

Materials and Methods. – Eggs and embryos of *Arbacia punctulata* and *Spisula solidissima* were treated at various stages with artificial sea water containing ^{3}H-uridine and cold thymidine for 30 min to 2 hr. A nonradioactive chaser was then applied for different intervals. Samples were fixed in glutaraldehyde or OsO_4 and embedded in glycol methacrylate or Epon for electron microscopic and autoradiographic study as reported previously.[4]

Results and Discussion. – Under the electron microscope, many dense bodies, "primary nucleoli," are found within the nucleus by the time of the 16-cell stage of *Arbacia* (2 hr after fertilization). They consist of dense fibrous material and are surrounded with chromatin fibrils. At the mesenchyme blastula stage (13 hr), larger nucleoli (approximately two per nucleus) become visible under the light microscope. These nucleoli are negative to cytochemical tests for RNA. By the time of gastrulation (15 hr), the RNA reaction becomes positive in the nucleolus, and ribosome-like particles become detectable within its peripheral portion. Later (24 hr), the nucleolus shows a typically high concentration of RNA and has a large number of ribosome-like particles throughout its area.

When thymidine is present in the medium, ^{3}H-uridine is exclusively incorporated into nuclear RNA throughout the development of *Arbacia* embryos. After 30 min of incubation in the isotope-containing medium, most of the label is confined in the nucleus. When the chase-incubation is extended to 2 or 4 hr, the cytoplasmic label increases at the expense of the nuclear label. During cleavage, sites of uridine incorporation are localized on the dispersed chromatin fibrils or the periphery of chromatin masses. At the mesenchyme-blastula stage, when nuclear RNA synthesis is significantly enhanced, labeled RNA begins to appear in the nucleoli. Later, in the nucleoli of the gastrula and pluteus, labeled RNA is first localized in the peripheral region and then becomes distributed throughout the whole nucleolar area. These results suggest that the chromatin associated with the periphery of the nucleoli is the initial site of nucleolar RNA synthesis.

Electron microscopic examinations of *Spisula* eggs also show the cleavage nucleoli to lack a granular component and to be composed mainly of fibrous material. At this time, ^{3}H-uridine incorporation into the nucleoli is negative. It is positive, on the other hand, in the chromatin material, indicating a synthesis of RNA there. After hatching (6 hr), the nucleoli incorporate ^{3}H-uridine and acquire granular components.

In both species the nucleoli appear as dense fibrous bodies during the cleavage stage. At this stage, soluble RNA and messenger RNA probably are synthesized in the chromatin material. Neither the appearance of ribosome-like particles in the nucleolus nor synthesis of nucleolar RNA begins until the early swimming stage.

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4.10 INTRANUCLEAR CRYSTAL WITHIN THE PHAGOCYTES OF THE OVARY OF *ARBACIA PUNCTULATA*

Shuichi Karasaki

During the course of an electron microscope study of the developmental process of *Arbacia punctulata* oocytes, the presence of protein crystal was found within the nuclei of the amoeboid phagocyte in the ovarian tissues.

In the present study, phagocytes containing such intranuclear crystalloids were frequently observed in the vicinity of fully grown oocytes. The frequency of amoeboid phagocytes containing crystalloids varied between 3 and 29% among four animals.

The intranuclear crystals range up to 5 μ in size and mostly exist as hexahedrons or tetrahedrons. Perl's Prussian blue reaction for ferric iron is clearly demonstrated in the crystals. An intense reaction to the mercuric bromphenol blue test indicates that the crystals also contain protein. Cytochemical tests for polysaccharides and nucleic acids are negative.

Under the electron microscope, thin sections embedded either in Epon or glycol methacrylate reveal a highly ordered periodicity in each intranuclear crystal. Sections of the crystals reveal dots or band patterns. Although the crystals have a high intrinsic density in unstained sections of aldehyde-fixed materials, the density is generally

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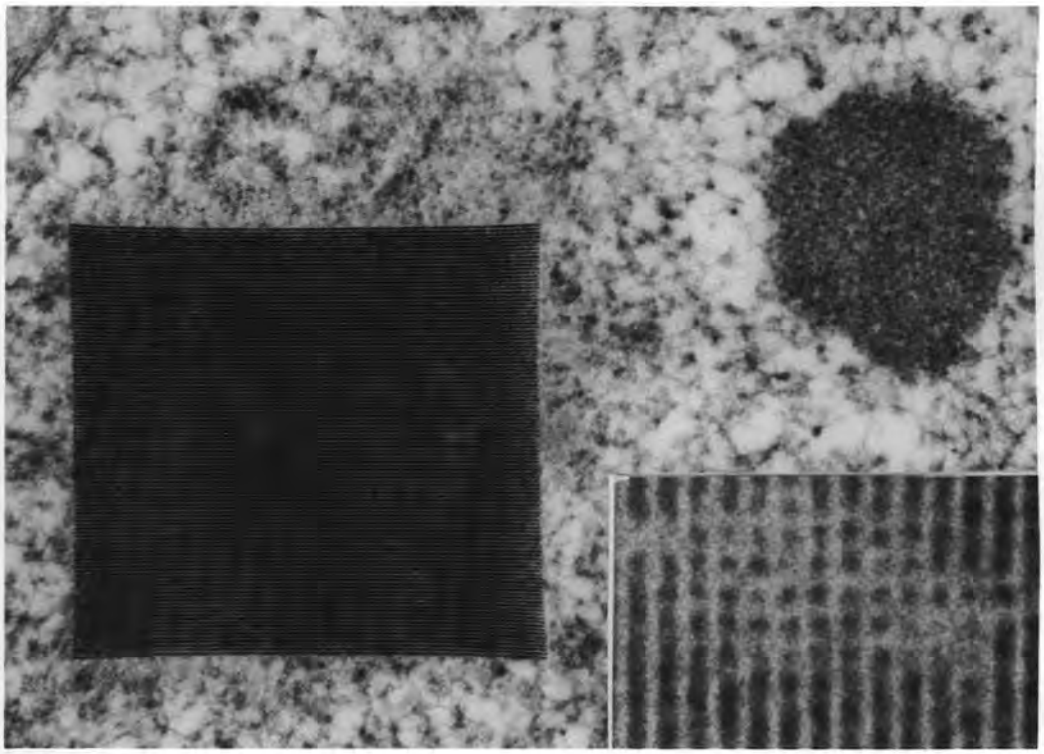

Fig. 4.10.1. An Electron Micrograph of a Thin Section of Phagocyte Nucleus. Intranuclear crystal and nucleolus. 50,000×. The inset in the right lower corner shows the high magnification of a portion of crystal. Spacing of dots 100 A. 400,000×.

increased significantly by uranium staining. The dot pattern packed in a square array is found in the square or rectangular profile of the crystals (Fig. 4.10.1). The mean of the center-to-center distances between the rows of dots is 101 A. Less frequently, an almost hexagonal array of dots is found in triangular profiles. The average distance between the dots is 102 A. These facts suggest that the basic unit of the crystal is spheroidal, with a diameter of about 100 A, and is closely packed in a face-centered cubic lattice form. This form may also be expected from the geometrical shapes of the crystal bodies themselves and from their isotropic nature under polarized light. This evidence suggests that the crystal material may be a variety of ferritin.

In the phagocyte containing a large intranuclear crystal, the nucleolus is specifically stained by pyronin and consists mainly of granular components, suggesting a high level of nuclear metabolism.

The function of the crystal of the iron-containing protein within the nucleus is unclear, but, in addition to the possibility of its being a segregated or stored protein, it may represent a synthetic product in the nucleus.

5. Radiation Microbiology

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Bacterial Genetics (5.2–5.4) H. I. Adler[a] L. J. Charamella Roy Curtiss III Alice A. Hardigree Claire M. Berg[b]	[a]Dual assignments. [b]ORINS Fellow.

5.1 RIBOSOMAL PROFILES OF RADIO-RESISTANT AND -SENSITIVE *ESCHERICHIA COLI*

G. E. Stapleton W. D. Fisher[1]
Annie S. Angel

Growth conditions that are conducive to development of radioresistance in *Escherichia coli* seem to involve a dissociation of macromolecular synthesis and cell division,[2,3] such that the presence of excess DNA, RNA, and protein per cell is characteristic of resistant cells as compared with their sensitive counterparts. Although the ratio of DNA to protein is relatively constant in this species and independent of growth conditions, the ratio of RNA to either protein or DNA in resistant cells is about twice that found in sensitive cells.

Cells in the late lag or early log phase of the growth cycle in broth are much more radioresistant than cells from elsewhere in the growth cycle. If glucose is supplied in the broth, however, stationary-phase cells are also radioresistant as compared with cells from the same phase that have not been supplied with this carbon source. Recent data of Adler and Hardigree[4] suggest that the radioresistance developed in the lag–early logarithmic phase is similar in several respects to that which develops in the stationary phase in glucose-broth cultures.

We have compared the sedimentation profiles of extracts of several strains of *E. coli* in the B-IV zonal ultracentrifuge described by Anderson *et al.*[5] This system permits the resolution of various soluble and particulate fractions of cell extracts.

The following comparisons were made: (1) *E. coli* (AB-1157) from stationary and early logarithmic phase nutrient broth cultures and (2) *E. coli* (X-160) from stationary nutrient broth cultures with and without the addition of 1% glucose.

Cell extracts were prepared in 0.01 *M* Tris buffer (pH 7.4) containing 0.01 *M* Mg^{2+}, by pressure disruption in the French press. The extracts were adjusted to approximately the same protein content, based on optical density at 280 mμ, then sedimented at 40,000 rpm for 2 hr in a sucrose gradient (10–30%), with a 55% sucrose cushion provided to prevent the heavier particles from moving to the rotor wall. By use of this technique the entire sample can be recovered by displacement of the rotor content after the run by pumping in 55% sucrose. The centrifuge used is provided with a system for continuous monitoring of the outflow

for absorption at 260 and at 280 mμ and for refractive index. These data are automatically recorded.

Figures 5.1.1 and 5.1.2 compare the absorption profiles (optical density at 260 mμ × $\frac{1}{5}$) for the cell extracts described. In both figures the solid lines represent the profiles for resistant cells, and the dashed lines the profiles for sensitive cells. The fractions are 40-ml cuts accumulated by displacement of the rotor contents. It is clear that the amount of RNA is greater for resistant cells than for sensitive cells. Of more interest is the finding that conditions which lead to radioresistance cause a shift in the ribosomal patterns. Sensitive cells contain predominantly large ribosomes, whereas resistant cells contain mostly smaller ones.

It is not clear what function small ribosomes play in development of radioresistance. However, it is of some interest that the 30S and 50S ribosomes are reported to be most labile, since they disappear upon magnesium starvation in this species,[6] and also upon postirradiation incubation in synthetic media.[7] It will be important to relate the lability of ribosomes to postirradiation phenomena related to repair or recovery of radiation damage in resistant and sensitive cells.

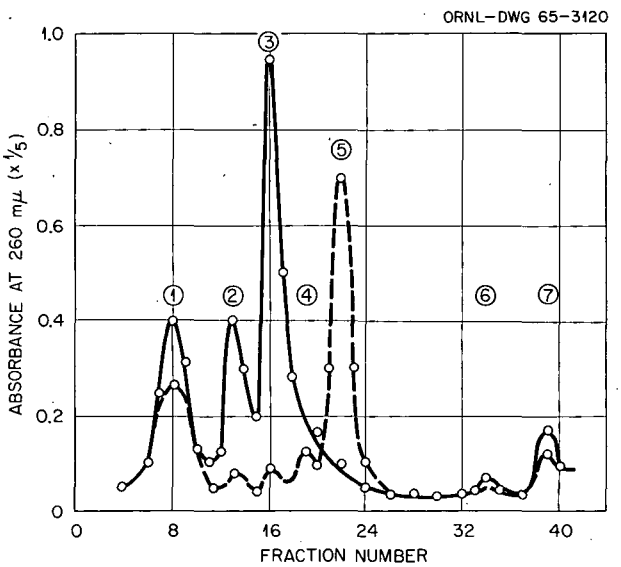

Fig. 5.1.1. Optical-Density Profiles from Pressure-Disrupted *E. coli* X-160. ----- cells grown in nutrient broth (Difco); ——— cells grown in same medium +1% glucose. The fraction numbers represent 40-ml gradient cuts. The peaks correspond to the following fractions: (1) soluble; (2), (3), (4), and (5) ribosomes, 30S, 50S, 70S, 100S, approximately; (6) submembrane; and (7) membrane-associated fraction.

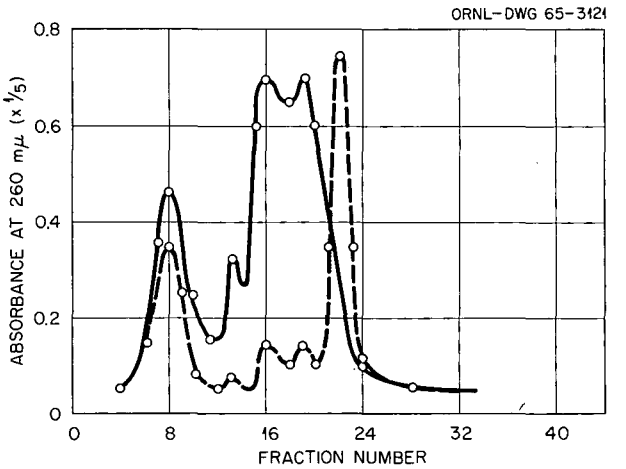

Fig. 5.1.2. Optical-Density Profiles from Pressure-Disrupted *E. coli* AB-1157. ----- cells grown to stationary phase in nutrient broth; ——— cells grown to early logarithmic phase in same medium.

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5.2 CAMPHOR-INDUCED RADIATION-RESISTANT MUTANTS OF *ESCHERICHIA COLI* K-12

H. I. Adler Alice A. Hardigree

In the past few years several mutations leading to increased ultraviolet and ionizing radiation

sensitivity in *Escherichia coli* have been reported. Gene loci have been established for some of these mutations by the application of conjugation techniques. Observation and study of mutations leading to enhanced radiation resistance have been, in contrast, relatively rare. The original isolation of the relatively radiation-resistant *E. coli* B/r from B (ref. 1) may now be regarded as a back mutation to wild type. This seems likely on the basis of recently obtained genetic information and the observation that most freshly isolated *E. coli* strains are like *E. coli* B/r in their radiobiological behavior.[2]

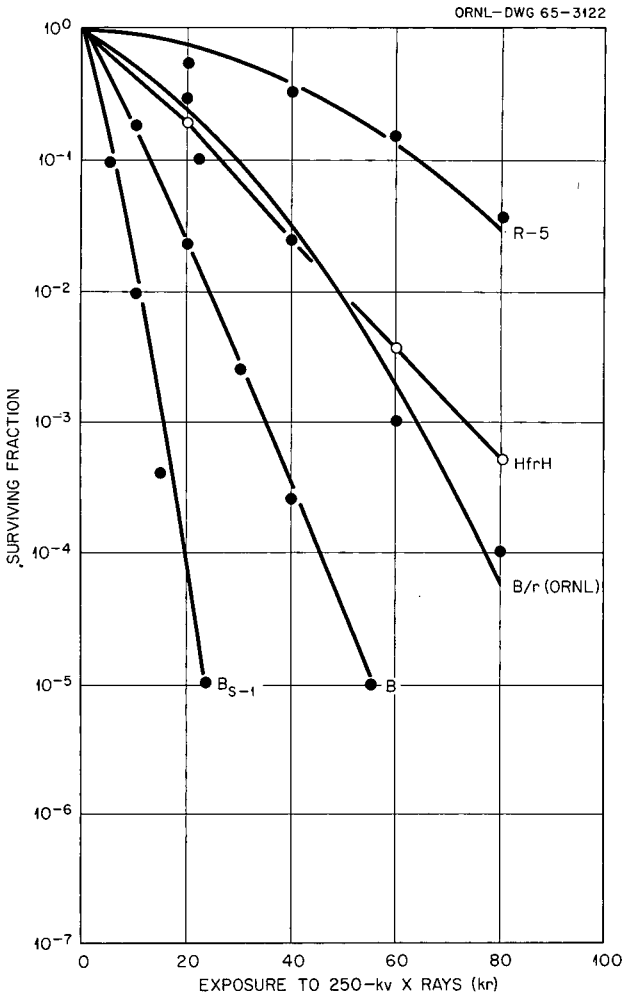

Fig. 5.2.1. The Radiation Resistance of R-5, a Mutant Recovered After Camphor Treatment of *E. coli* K-12 HfrH. Other commonly used *E. coli* strains are included for comparison. All cultures were grown in nutrient broth and plated on nutrient agar after irradiation.

We wanted to find out if mutant strains with greater radiation resistance than *E. coli* B/r could be obtained and genetically characterized. Zelle and Ogg[3] reported that camphor could induce such mutants in an auxotrophic derivative of *E. coli* B/r, but their genetic studies were hampered by the restrictions imposed on conjugation experiments by strains derived from *E. coli* B. We, therefore, have attempted to isolate radiation-resistant mutants from several *E. coli* K-12 strains. The radiation resistance of most K-12 strains is similar to that of *E. coli* B/r.

Approximately 10^8 cells of each strain tested were spread onto the surface of a nutrient agar plate. A small block of camphor was placed on the agar near the center of the plate. After several days of incubation, small, discrete colonies appeared close to the camphor block. These were picked, restreaked, and assayed for x-ray sensitivity. One hundred seventy isolates from two K-12 F^- strains did not yield any organisms of altered radiation sensitivity. However, 12 mutants were obtained from a group of 73 isolates of the Hfr Hayes strain of *E. coli* K-12. Of these 12, 6 are significantly more radiation resistant than the parental strain and 6 are more sensitive. The behavior of one of the resistant strains (R-5) is being examined in greater detail. It is unusually resistant to both ionizing (Fig. 5.2.1) and ultraviolet (2537 A) radiation. It is a high-frequency donor of genetic material and injects its genes into F^- strains in the same order and at the same frequency as the parental Hfr Hayes culture. To date we have not been able to establish a locus responsible for the high level of radiation resistance.

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5.3 MUTANT ISOLATION BY A D-CYCLOSERINE ENRICHMENT CYCLING TECHNIQUE

Roy Curtiss III Claire M. Berg
L. J. Charamella

D-Cycloserine (2×10^{-3} *M*) is as efficient as penicillin (2000 units/ml) for mutant enrichment in *Escherichia coli* and should be useful for mutant isolation in penicillin-resistant microorganisms. *E. coli* cells growing at 37°C in mineral-salts–glucose medium with vigorous aeration are lysed at maximum exponential rates by 10^{-4} to 10^{-2} *M* D-cycloserine. At concentrations above 2×10^{-2} *M*, D-cycloserine is bacteriostatic. Spontaneous mutations to D-cycloserine resistance occur in discrete steps. First- and second-step D-cycloserine–resistant mutants are lysed at maximum exponential rates by D-cycloserine concentrations of 10^{-3} *M* or above. D-Alanine at low concentrations (5×10^{-6} to 5×10^{-5} *M*) and L-alanine at high concentrations (10^{-4} to 10^{-3} *M*) cause partial to complete competitive reversal of lysis by 10^{-4} *M* D-cycloserine. Therefore, low cell densities must be used to prevent a decrease in the exponential rate of lysis of liberated D- and L-alanine.

The mutant enrichment procedure developed is as follows:

1. Treat with mutagen.
2. Grow treated suspension with aeration in minimal media supplemented to permit growth of desired mutant type(s).
3. Wash cells by centrifugation or by collection on membrane filters and resuspend in media which will only support growth of nonmutants.
4. Aerate to starve mutants and to allow nonmutants to begin growing (1–3 hr).
5. Prepare cycloserine freshly in 0.1 *M* phosphate buffer, pH 8, and add to culture (10^7 cells/ml) to 2×10^{-3} *M*. Continue aerating 2–4 hr, depending on growth rate.
6. Collect unlysed cells on membrane filters and wash.
7. Repeat steps 2–6, or isolate mutants by replica plating.

This method can be used to enrich for several different mutant types concurrently.

5.4 AN EFFECT OF MITOMYCIN C ON FILAMENTOUS FORMS OF *ESCHERICHIA COLI*[1]

H. I. Adler Alice A. Hardigree

Cells of certain mutant strains of *Escherichia coli* grow into long, multinucleate filaments after exposure to radiation. The growing filaments synthesize DNA, RNA, and protein, but are not capable of cell division, and eventually lyse. If the filaments are exposed to pantoyl lactone or a temperature of 42°C, they begin to divide and produce normal-length cells. These cells continue to grow and divide even after the removal of the division-stimulating agent.

We wanted to know if cell division could be induced in filaments in which DNA synthesis and nuclear division had been inhibited. To this end, we have treated populations of radiation-induced filaments with the antitumor agent mitomycin C. This agent interferes with DNA synthesis in filaments, and we have observed, by phase microscopy, that nuclear material is confined to the central region of mitomycin C–treated filaments. Although the literature contains data suggesting that mitomycin C treatment leads to the solubilization and loss of DNA fragments from bacterial cells,[2] we do not observe these phenomena under our experimental conditions.

Many hundreds of mitomycin C–treated filaments have been observed microscopically after exposure to the division-stimulating agents, but in no case have we observed any indication of cell division. The fact that no cytokinesis is observed in such filaments suggests that nuclear material plays an active role in the cross-septation process. Furthermore, it seems likely that nuclei must be located on both sides of the point at which cross septation is to occur. We have never observed a case in which cross septation results in the formation of an enucleate cell. It may be that the division-promoting agents act directly on the nuclei which, in turn, control the cross-septation process. Mitomycin C, by interfering with nuclear synthesis and replication, prevents the activity of the division-promoting agents.

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6. Mammalian Genetics

Section Chief – W. L. Russell

Genetic Effects of Radiation in Mice (6.1, 6.2)

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Effects of Radiation on Mammalian Gametogenesis (6.5)

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

W. L. Russell Elizabeth M. Kelly

The results obtained to date, Table 6.1.1, on specific locus mutations in adult female mice exposed to fission neutrons from the Health Physics Research Reactor[1] show a tremendous effect of the interval between irradiation and conception on mutation frequency.

Neutron irradiation has proved to be especially suitable for this study for the following two reasons. First, with the dose used at the higher dose rate, the RBE for neutron compared to x-ray effects on fertility in females is only of the order of 1, whereas the RBE for genetic effects has turned out to be much higher. Thus it has been possible to give an acute neutron dose which will allow fertility to persist well beyond seven weeks and which, at the same time, will produce enough mutations for a reliable measure of mutation frequency. Second, at dose rates which, with gamma irradiation, are low enough to give fertility beyond seven weeks, the mutation frequency, even at high doses, is so low, owing to the large dose-rate effect with gamma irradiation of oocytes, that measurement of mutation frequency is difficult. With fission neutrons, on the other hand, although the data in Table 6.1.1 show strong evidence of some dose-rate effect on mutation in oocytes, the effect is not as great as that with gamma rays. Consequently, large numbers of mutations are still observed in the early interval after irradiation,

Table 6.1.1. Frequency of Specific Locus Mutations at Two Intervals After Irradiation of Female Mice with Fission Neutrons

Dose[a] (rads)	Dose Rate (rads/min)	Interval Between Irradiation and Conception			
		Up to Seven Weeks		More than Seven Weeks	
		Number of Offspring	Number of Mutations at Seven Loci	Number of Offspring	Number of Mutations at Seven Loci
63	0.17	46,301	22	73,711	0
63	79	43,000	37	37,308	0

[a]Air dose. Dose to gonads estimated to be somewhat lower. Includes a gamma component equal to approximately one-seventh of the neutron component.

Table 6.1.2. Frequency of Specific Locus Mutations After Irradiation of Female Mice with ^{137}Cs Gamma Rays

Dose (r)	Dose Rate (r/min)	Interval Between Irradiation and Conception			
		Up to Seven Weeks		More than Seven Weeks	
		Number of Offspring	Number of Mutations at Seven Loci	Number of Offspring	Number of Mutations at Seven Loci
258	0.009	8,439	1	18,735	0
400	0.009	29,325	3	22,807	1

even when the dose rate is lowered to the level at which it will give extended fertility compared with acute irradiation.

The absence of any mutations so far in the 111,019 offspring obtained in the later time interval after irradiation (see Table 6.1.1) is remarkable. Even the upper 99% confidence limit of this zero frequency is below the spontaneous mutation rate in male mice. (A reliable value for the spontaneous rate in females is not yet available.) It is of great importance to find out whether this marked effect is due to a low mutational sensitivity of oocytes in early follicle stages, to an efficient repair mechanism in these stages, or to cell selection. If it is due to selection, the mechanism could conceivably be the survival of only those oocytes that escaped having a proton track pass through their genetic material. In this case, the effect would not be seen with x and gamma rays. The data shown in Table 6.1.2 argue against this view to some extent. Although the number of mutations is low, because of the low dose rate, there is some indication that an effect of the irradiation-conception interval on mutation frequency may occur with gamma rays as well as with fission neutrons.

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6.2 ANALYSIS OF STRAIN DIFFERENCES IN THE REPRODUCTIVE CAPACITY OF IRRADIATED FEMALE MICE

U. H. Ehling

Highly significant strain differences in the reproductive capacity of irradiated female mice have been reported.[1] It is remarkable that DBA females, after exposure to 50 r, produced more than twice as many offspring per female as did irradiated SEC females (DBA, 22.5 ± 1.6; SEC, 11.0 ± 0.9), especially since DBA controls produced little more than half as many offspring as did SEC controls (DBA, 40.4 ± 3.7; SEC, 73.4 ± 0.4). Crosses and backcrosses between SEC and DBA strains were made to determine the cause of the intraspecific variation of the radiation response.

The procedures for and initial results of evaluating the strain differences have been described in an earlier report.[2]

The reproductive capacity of hybrid females over a period of 210 days is summarized in Table 6.2.1. The unirradiated hybrid females of reciprocal crosses produced the same number of offspring and litters per female. In contrast to the controls, the irradiated females of reciprocal crosses produced a significantly different number of offspring and litters per female ($P < 0.01$). This difference is probably attributable to a maternal effect. The evaluation of the radiation response of backcross females that is now in progress will have to take this maternal influence into consideration.

Table 6.2.1. Reproductive Capacity of Hybrid Females over a Period of 210 Days

Genotype	Doses (r)	Offspring per Female	Litters per Female
(SEC × DBA)F_1	0	71.8 ± 1.7	9.0 ± 0.2
	50	15.3 ± 0.7	2.8 ± 0.2
(DBA × SEC)F_1	0	71.4 ± 2.0	8.8 ± 0.1
	50	19.7 ± 1.5	3.7 ± 0.3

References

[1]U. H. Ehling, *Radiation Res.* **23**, 603–10 (1964).
[2]U. H. Ehling, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1964,* ORNL-3700, p. 85.

6.3 SPONTANEOUS MUTABILITY IN THE MOUSE: CONCLUSIONS FROM MOSAIC MUTATIONS

Liane B. Russell

Over the years, fewer than 20 cases of possible mouse mosaics, including only 4 with germinal involvement, have been reported from other laboratories. The work of this laboratory has provided unparalleled opportunity for the detection of mosaics, since, in the course of specific-locus mutation rate studies, many thousands of mice are observed that should start life heterozygous for seven recessives, five of them affecting coat color. Because some of the markers are linked, and because, at some of the loci, the test-stock allele is different from that to which most mutations occur, the setup is also favorable for subsequent genetic testing of the presumed mosaics.

Analysis is only partially completed. In the sample so far tabulated, there were 115 "mottled" mutants. In 21 of these, mottling was found to have a cause other than mosaicism for one of the five coat-color loci (e.g., sex-linked genes, X-autosome translocations, etc.). Among the remaining 94 presumed fractional mutants, there were 43 in which mosaicism could be demonstrated by genetic tests.

The incidence of mosaics in controls was at least as high as that among offspring of any of a number of different irradiated germ-cell stages (see Russell[1] for one of these stages). There is thus no evidence that radiation causes partial mutations. As pointed out by Muller *et al.*,[2] this would mean, in the case of postmeiotic germ-cell stages in the male, that radiation affects both strands of the DNA molecule.

In 43 gonosomic mosaics whose breeding results were analyzed, there was no evidence for selection against mutant cells in the gonad or in the progeny. The progeny ratios can, therefore, be used with confidence in calculating cell-type proportions. The distribution of these mosaics with respect

to proportion of mutant cells in germinal tissue had a mean of 48.3%. We, therefore, consider these animals to be a population of half-and-half mosaics. Such mosaics could result from "spontaneous" change in one strand of DNA any time after the last premeiotic duplication (primary spermatocyte) and before the duplication that presumably occurs in the zygote. (Or, providing that *both* strands of DNA are affected, they could result at the time of the first cleavage or in the two-cell stage.) Mutations occurring before the last premeiotic duplication (e.g., gonial stages) will give whole-body mutants, regardless of whether one strand or both strands of DNA are affected. Since spontaneous fractional mutations are probably as frequent as spontaneous whole-body mutations, it may be concluded that the stages between the last premeiotic duplication and the first cleavage are much more spontaneously mutable than are any other stages.

Analysis of the 43 gonosomic mosaics also provides information on the cell lineage of the gonad primordium.[3] The distribution of cell-type proportions is broad, and the results fit one of two alternatives. (1) The gonad primordium is set aside as a random assortment of a *small* number of cells, averaging about 5, from a cell population that is 50% mutant. The primordial cells may contribute unequally to the mature germinal tissue. (2) Some segregation into cell lineages has occurred before the time when the gonad primordium is set aside.

References

[1]W. L. Russell, pp. 257–64 in *Genetics Today*, vol. 2, ed. by S. J. Geerts, Pergamon, Oxford, 1965.

[2]H. J. Muller, E. Carlson, and A. Schalet, *Genetics* **46**, 213 (1961).

[3]Liane B. Russell, pp. 153–81 in *The Role of Chromosomes in Development*, ed. by M. Locke, Academic, New York, 1964.

6.4 PATTERN OF DNA SYNTHESIS IN X-AUTOSOME TRANSLOCATIONS IN THE MOUSE

E. H. Y. Chu Liane B. Russell

(For the text of this report see 1.21 in the section on Cytology and Genetics.)

6.5 EFFECT OF 25-r X RAYS ON FERTILITY AND OOCYTE NUMBER OF 10-DAY-OLD FEMALE MICE

E. F. Oakberg Jo K. Henderson
Evelyn Clark Susie H. Heckman

Early oocyte stages of 10-day-old female mice have been shown to be extremely sensitive to radiation, with LD_{50}'s of 8.4 and 9.4 r for stages I and II respectively.[1] Nevertheless, L. B. Russell obtained an average of nine litters from females given 25 r at 10 days of age; yet, oocyte survival after this dose was only 3% for stage I and 9% for stage II.[1] The present experiment was designed to elucidate this apparent paradox between oocyte killing and fertility.

Female mice were distributed at random among four experimental groups: (1) control, mated; (2) control, not mated; (3) irradiated, mated; and (4) irradiated, not mated. Mice were exposed to 25 r of 250-kv x rays at a dose rate of approximately 9 r/min when 10 days old, and returned to their mothers. When 8 weeks old, females of groups 1 and 3 were placed with 101 × C3H males and left until termination of the experiment. Males that died were replaced. All young were recorded and discarded at birth. Three controls were killed at 10 days, and three control and three irradiated mice were killed at 13, 28, and 56 days of age. Three mice of each group were killed at 103, 165, 220, and 320 days, and from groups 1 and 2 at 550 days. Ovaries were fixed in Zenker-formol, sectioned serially at 7 μ, and the number of stage I and stage II oocytes counted in every tenth section.

Eighteen control females had a total of 301 litters and 1882 young, or an average of 16.7 litters and 104.6 young per female. Thirteen irradiated females had 92 litters and 475 young, or an average of 7.1 litters and 36.5 young per female. Oocyte survival and changes in number of oocytes with age are given in Table 6.5.1.

As observed previously, the reduction in number of litters from 17 to 7 is not as large an effect as one might expect from reduction of number of oocytes to 7% of control. However, there appears to be no conservation of oocytes by the irradiated female, that is, loss with age occurs at about the same rate in both irradiated and control series. Also, there is no difference between mated and virgin females, indicating that only late follicle

Table 6.5.1. Effect of 25 r at 10 Days of Age on Oocyte Numbers in Mated and Virgin Female Mice

Age (days)	Control		Irradiated	
	Mated	Virgin	Mated	Virgin
10		8980		
13		9380		620
28		8280		310
56		6060		160
103	5530	6190	140	100
165	4000	3800	20	30
220	2610	2680	0	3
320	1780	1390	0	0
550	350	180		

stages undergo atresia in pregnancy. Follicles develop to these stages, then degenerate.

Radiation-induced sterility cannot be considered as an enhanced aging phenomenon, since the mechanism involved is different in irradiated and control mice. Irradiated females become sterile when the oocyte supply is exhausted; when old control females become sterile, they still have adequate numbers of oocytes. Thus, in the normal female mouse, sterility of old age must result from physiological mechanisms affecting ovulation, implantation, or maintenance of the embryo.

References

[1]E. F. Oakberg, *Proc. Soc. Exptl. Biol. Med.* **109**, 763–67 (1962).

7. Human Cytogenetics

M. A Bender[a]	F. M. Faulcon
J. G. Brewen	Katherine P. Jones
P. Carolyn Gooch[a]	W. H. Lee
Gregorio Olivieri[b,c]	F. G. Pearson
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7.1 VARIATIONS IN X-RAY-INDUCED CHROMOSOMAL ABERRATIONS IN HUMAN LEUKOCYTES IN RELATION TO THE CELL CYCLE

J. G. Brewen

It has been well established that cells' response to ionizing radiation varies with their position in the cell cycle at the time of irradiation. Work with the classical plant materials shows that both the types and the frequencies of x-ray-induced chromosomal aberrations differ with respect to the cell cycle. It has also been shown that mammalian cell x-ray survival varies at different stages of the cell cycle. I have studied the types and frequencies of x-ray-induced chromosomal aberrations observed at the first postirradiation mitotic metaphase of human peripheral leukocytes irradiated at different stages in the cell cycle.

Tissue cultures of peripheral leukocytes were irradiated at various times between their initiation and fixation. The cultures were pulse labeled with tritiated thymidine in order to distinguish DNA-synthesizing (S) cells from the pre-DNA-synthesizing (G_1) and post-DNA-synthesizing (G_2) cells. The

Table 7.1.1. Pooled Frequencies of Aberrations Observed in Human Leukocytes in Different Stages of the Cell Cycle After 100 r of X Rays

Treatment	Percentage of					
	Chromatid Deletions	Isochromatid Deletions	Chromatid Exchanges	Chromosome Deletions		Two-hit Chromosome Aberrations
				Terminal	Interstitial	
Control	1.2	0.0	0.0	0.0	0.0	0.0
100 r, G_1	0.8	0.0	0.0	6.0	2.0	8.8
100 r, early S	4.3	1.0		0.0	0.0	0.3
100 r, mid–late S	3.8	2.6	0.7	0.0	0.0	0.0
100 r, G_2	19.2	2.0	5.1	0.0	0.0	0.0

x-ray exposure was 100 r in all experiments. Metaphase figures were scored for chromosomal aberrations and for the presence or absence of silver grains in their autoradiographs. Whether nonlabeled (non-S) cells were in G_1 or G_2 at the time of irradiation was determined by whether the irradiation was performed before the time such cells are known to begin DNA synthesis.

Table 7.1.1 summarizes the data obtained in the experiments. G_2 is obviously the most x-ray-sensitive stage of the cell cycle, and S the least sensitive. The variation in the number of two-hit chromosomal aberrations is interpreted as being the result of differences in the size or number of sites within an interphase nucleus within which exchanges can occur. Thus G_1 and G_2 cells have more or larger sites than S cells. The increase in terminal deletions in G_2 cells is believed to be caused by the coiling of the chromosomes moving broken ends apart before restitution can occur. The similar frequencies of terminal deletions in S and G_1 cells argue against changes in the sensitivity of the chromosome strand, as do the similar frequencies of isochromatid deletions in S and G_2 cells. If chromosome sensitivity to x rays changed during the cell cycle, the frequencies of all types of aberrations would be expected to vary with position in the cell cycle, each type remaining a constant proportion of the total if allowance is made for the multiple-hit kinetics for exchanges.

7.2 ELECTRON MICROSCOPIC STUDY OF C-METAPHASE CHROMOSOMES ISOLATED FROM CHINESE HAMSTER FIBROBLASTS

Hiroshi Yamamoto

Most information about the ultrastructure of chromosomes is based on studies of plant cells, animal germ cells, and cells with atypical chromosomes, such as the giant polytenic chromosomes of Diptera and the lampbrush chromosomes of amphibian oocytes. I have investigated the ultrastructure of the chromosomes of tissue-cultured fibroblasts from a Chinese hamster cell line, both to learn something of the structure of mammalian somatic chromosomes and to evaluate the use of the electron microscope as an aid in mammalian karyotype analysis.

A "pseudodiploid" Chinese hamster cell line (C-125) was cultured by conventional methods. After 78 hr of incubation, when the cells were in the logarithmic growth phase, they were treated with colchicine (10^{-4} *M*) for 5 hr. Hypotonic treatment, where given, was with 0.7% sodium citrate for 20 min. C-Metaphase chromosomes were isolated by the glacial acetic acid method. Prefixation trypsin treatment was with 0.5 mg of enzyme per milliliter of 0.007 *M* phosphate buffer at pH 7.2 for times ranging from 5 to 120 min. For thin sections, cells were fixed in 4% $KMnO_4$ for 2 hr and embedded in epoxy resins. These materials were examined by phase contrast and fluorescence microscopy as well as with the electron microscope (accelerating voltages of 50 and 100 kv).

In the fluorescence microscope, isolated chromosomes stained with acridine orange fluoresced with a brilliant green color and were surrounded with an orange-fluorescing material which might have been adhering cytoplasm. After 4 to 12 days' treatment with acetic acid, swelling and uncoiling of the chromosomes could be seen. Loss of some chromosomal material is suggested by rough measurements of electron density per unit area or per isolated chromosome. Measurements were made immediately after isolation and after 12 days' acid treatment. Without hypotonic prefixation treatment, chromosomes fixed in $KMnO_4$ appeared homogeneous. After hypotonic citrate prefixation treatment, however, the chromosomal structure became looser, and threads about 500 and 1200 A in diameter appeared. Electron micrographs of serial sections of the centromere areas of $KMnO_4$-fixed isolated chromosomes clearly show a multistranded and spiralized configuration. When isolated chromosomes were fixed in saturated uranyl acetate, the centromeres clearly showed 60, 150–200, and 450–500 A threads. In the centromeres of chromosomes fixed and stained with acetoorcein, only spiralized 480-A threads could be seen. Threads of these same sizes were also seen in the distal, medial, and proximal regions of the arms of these chromosomes. Chromosomes treated with trypsin for 5 min showed uncoiling and swelling of both long and short chromosome arms, and both half and quarter chromatids appeared. Some of the chromomeres, which consist of tightly coiled convolutions of 500-A threads, remained tightly coiled and electron dense. After 20–30 min of trypsin treatment the chromosome arms showed uncoiling and swelling, but the regions proximal to the centromere remained densely packed. Figure 7.2.1 shows these features in a chromosome treated for 1 hr. After 90 min of trypsin treatment the

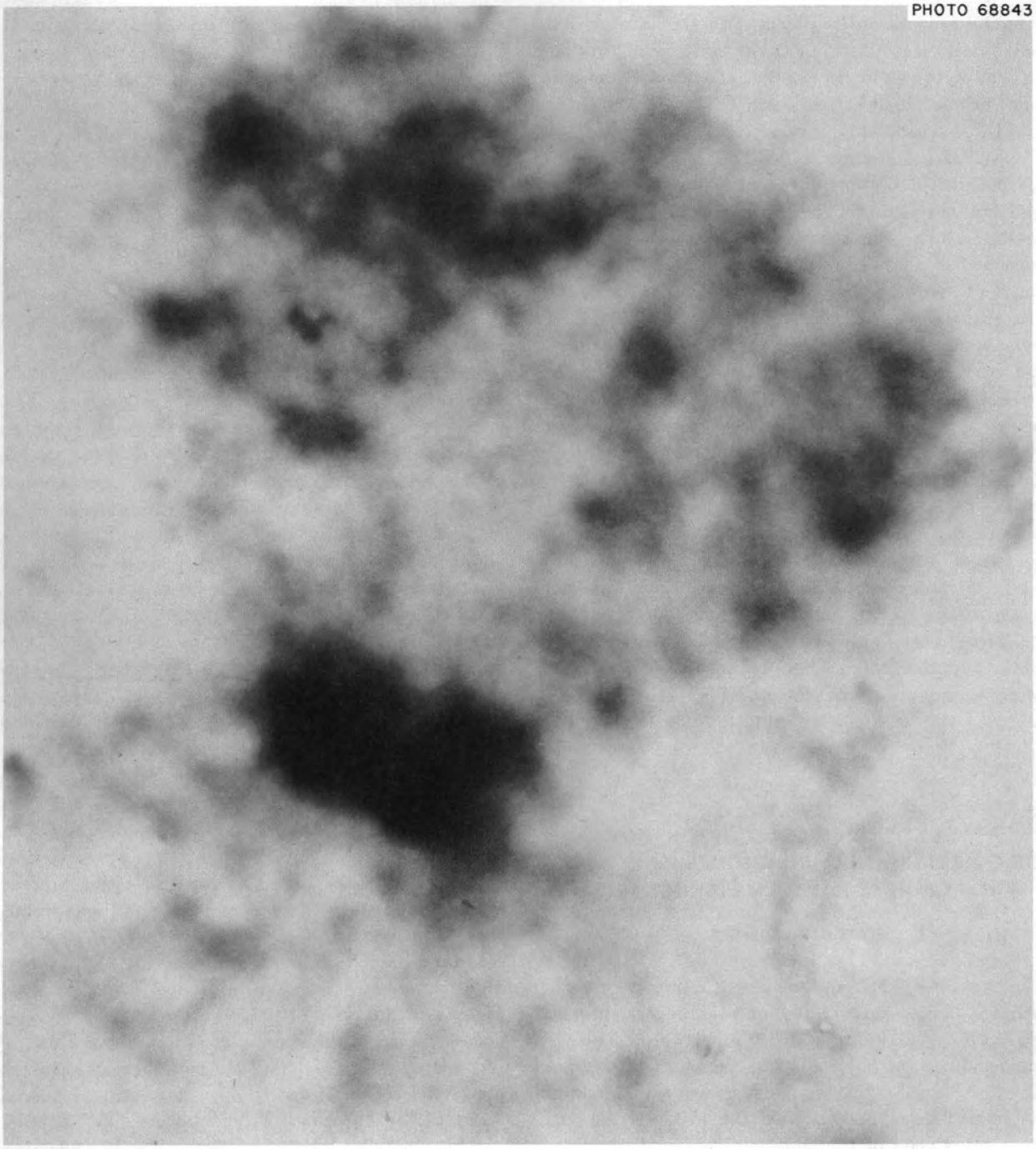

Fig. 7.2.1. Air-Dried Trypsin-Treated Isolated Colchicine-Metaphase Chromosome. Trypsin treatment: 0.5 mg/ml 0.007 *M* phosphate buffer, pH 7.2, for 1 hr at room temperature. Electron micrograph shows two chromatids (short arms). 65,000×. Reduced 7%.

uncoiling and swelling of the arm portions were more advanced, but the proximal regions remained dense. The data suggest that the chemical nature of the proximal regions is different from that of the distal regions. Either the protein of the proximal regions is largely nonhistone, or the proximal region histones have few lysyl and arginyl residues, or have their lysyl and arginyl residues adjacent to prolyl, aspartyl, and glutamyl residues.

7.3 CHROMOSOME ABERRATIONS IN PERSONS IRRADIATED IN A URANIUM CRITICALITY ACCIDENT ON JULY 24, 1964

P. Carolyn Gooch

A uranium criticality accident occurred on the evening of July 24, 1964. Blood samples were obtained from six individuals who were believed to have been exposed to small radiation doses. Unfortunately, no notification of the accident was received in Oak Ridge, and it was not until after an article appeared in a local newspaper on July 26, 1964, that the blood samples were obtained. A seventh individual, heavily exposed, had died before anyone from Oak Ridge reached the scene, and postmortem blood samples from him were not satisfactory. Two of the remaining six men were hospitalized. Blood samples were obtained on July 27 (three days after the accident). These cultures were not satisfactory. Blood samples were obtained from the other four men on July 28. A second set of samples was obtained from the first two men on July 31. Material suitable for chromosome aberration analyses was obtained from the four-day samples (of B, C, M, and Sp), and from one of the one-week samples (H). The other one-week sample (Sm) was unsuccessful, possibly because of the stressful situation in which this individual found himself during this period of uncertainty about the magnitude of the dose he had received. Additional samples were obtained from the two most heavily exposed survivors at $2\frac{1}{2}$, $3\frac{1}{2}$, $5\frac{1}{2}$, and 10 weeks postaccident.

Studies of previous accidental radiation exposures have shown that there is little difference in aberration rates during the first four postirradiation weeks. Thus, all except the $5\frac{1}{2}$- and 10-week samples may be regarded as fair measures of the induced aberration frequency in the present cases. The results of the aberration analyses are shown in Table 7.3.1. Very little information is available about the doses which the men received. The low aberration rates seen in the samples for B, C, M, and Sp are, however, consistent with what little is known about the accident. The results for H and Sm are consistent with their having, as was believed at the time, received higher doses. As H was not present in the accident area when it occurred, it was at first believed that he could only have received gamma irradiation from the fission products left after the criticality. The relatively low frequency of two-break aberrations in the early samples of his leukocytes agrees with this idea. The relatively high frequencies of two-break aberrations in relation to the frequencies of one-break aberrations in the samples from Sm, however, suggest that he received an appreciable neutron exposure. Physical evidence obtained after this biological observation was made bears out this theory.

It is possible to estimate the doses received by the individuals involved in the accident from the aberration frequencies observed. The coefficients of aberration production used (obtained from previous experimental irradiations of whole human blood) are: for gamma rays, 0.09×10^{-2} deletion per cell per rad, and 0.6×10^{-5} dicentric per cell per rad^2; for fission spectrum neutrons, 0.45×10^{-2} deletion per cell per rad, and 0.55×10^{-2} dicentric per cell per rad. Table 7.3.2 gives the doses calculated for the exposed men, assuming either gamma irradiation only or neutron irradiation only, for the blood samples taken up to $3\frac{1}{2}$ weeks. The data for H and Sm are sufficient to allow an estimate to be made of their actual mixed irradiation exposures. The best estimates are: for H, 35 rads of gamma plus 1 rad of neutrons; for Sm, 5 rads of gamma plus 3 rads of neutrons. It should be noted, however, that such estimates assume homogeneous exposure over the entire body; both of these men probably actually received inhomogeneous exposures. The estimates, therefore, are what might be termed "homogeneous, whole-body equivalents." The finding of a six-break tetracentric aberration in the $5\frac{1}{2}$-week sample from H suggests that his exposure was, in fact, quite inhomogeneous.

Table 7.3.1. Chromosomal Aberrations in Peripheral Leukocytes of Men Exposed in a Uranium Criticality Accident on July 24, 1964

Individual	Time of Sampling	Cells Scored	Chromosome-Type Aberrations				
			Deletions	Dicentrics	Rings	Exchanges	Breaks[a] (%)
H	1 week	150	7	0	0	0	4.7
	2½ weeks	100	2	0	0	1	2.0
	3½ weeks	100	4	1	0	0	6.0
	5½ weeks	100	3	4[b]	0	0	11.0
	10 weeks	100	0	1	0	1	2.0
Sm	2½ weeks	150	2	3	0	3	5.3
	3½ weeks	100	0	2	0	1	4.0
	5½ weeks	100	0	0	1	1	2.0
	10 weeks	100	3	0	0	0	3.0
B	4 days	150	3	0	0	1	2.0
C	4 days	100	2	0	0	0	2.0
M	4 days	150	0	0	0	0	2.7
Sp	4 days	150	2	0	0	0	2.0

[a]Exchanges not included in calculations because of the subjectivity in scoring that type of aberration.
[b]Including one tetracentric counted as three dicentrics.

Table 7.3.2. Dose Estimates from Aberration Data Given in Table 7.3.1

Individual	Dose Estimates Assuming Only Gamma Irradiation (rads)		Dose Estimates Assuming Only Neutron Irradiation (rads)	
	Deletions	Dicentrics	Deletions	Dicentrics
H	38.5	22.0	7.69	0.52
Sm	6.1	58.0	1.2	3.6
B	19.4		3.9	
C	19.4		3.9	
M		46.5		2.4
Sp	11.7		2.4	

8. Mammalian Recovery

C. C Congdon[a]

Secondary Disease (8.1–8.4)

C. C Congdon[a]
D. C. Swartzendruber[a]
W. H. McArthur[b]
A. L. Kretchmar[c]
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Recovery in Vitro (8.5)

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Hemopoietic and Lymphatic Stem Cells (8.6)

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Bone Marrow Tissue Culture (8.7)

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Animal Testing Program (8.8)

M. L. Simmons
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8.1 FACTORS AFFECTING MORTALITY FROM SECONDARY DISEASE

C. C Congdon[1]

Introduction. – Several years ago a number of studies on modifying mortality from secondary disease in radiation chimeras were carried out in our laboratory and elsewhere. It is now apparent that the interpretation of the results of the earlier studies is complicated by the variable mortality from secondary disease that occurs in supposedly standardized control experiments.

In the present work we decided to restudy the 90-day mortality to determine whether variables that minimize and those that maximize 90-day mortality could be identified. Experiments were designed in which the donor and host mouse strains, host age, and radiation exposure were all kept constant. Sex, age of donor cells, day of injection, and injected cell dose were varied.

Three separate designed experiments were carried out, and all showed variance in mortality among cages of five animals greater than would be expected from a binomial distribution of deaths among the different cages. The cause of this "cage effect" in death of mice is not known. Of 1429 experimental animals given foreign bone marrow after lethal irradiation, about 50% died in 90 days (Fig. 8.1.1). Sample studies on the survivors showed that their red blood cells were all of donor type. Bone marrow cell dose (10 or 50 million), day of injection (0 or 3), and sex failed to affect the pattern of cumulative mortality.

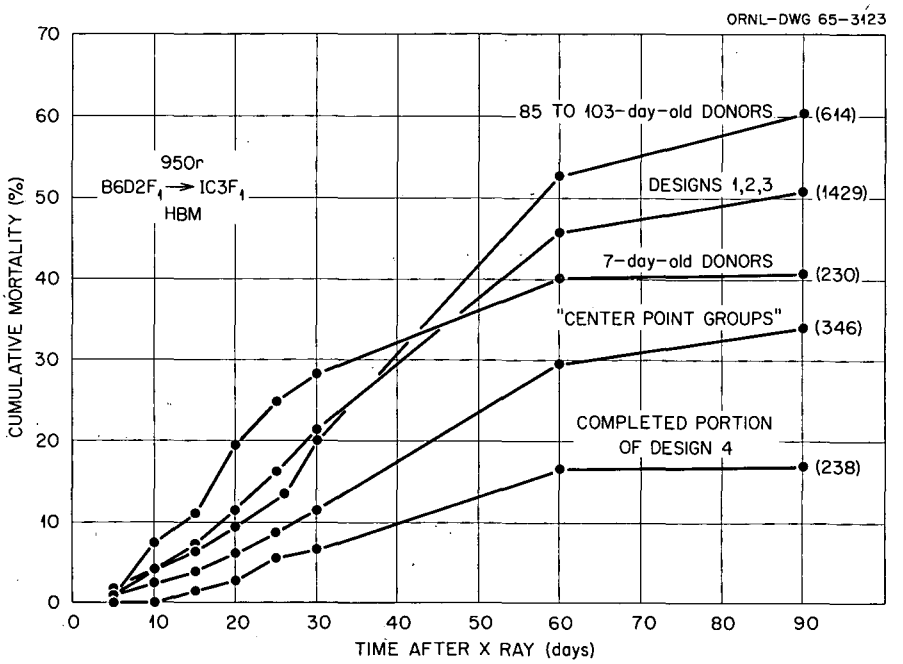

Fig. 8.1.1. Ninety-Day Cumulative Mortality in Different Groups of Radiation Chimeras.

When donor marrow cells were taken from mice 7 and 14 days of age, there was 10 to 20% less mortality than when the cells were taken from mice 21 or 85 to 103 days of age. The least 90-day mortality from secondary disease (about 35%) occurred in a special "center-point group" of mice that received 30 million bone marrow cells from 44- to 61-day-old donors on day 1.5 after 950 r of x rays (Fig. 8.1.1). There was also less variance in mortality among cages ("cage effect") in the center-point group.

The present report concerns a new study (design 4, Fig. 8.1.1) based on reduced 90-day mortality seen with 7-day-old donor marrow and the levels of other variables used in the center-point group. In design 4 the bone marrow donor age was 3, 10, or 17 days; the day of injection was 1, 1.5, or 2; and the dose of cells was 20, 30, or 40 million.

Results and Discussion. – The cumulative mortality for 238 mice in design 4 was considerably lower than that seen for comparable large groups in our earlier studies. Tests on the surviving mice for chimerism are not completed. If all the 90-day survivors are chimeras and if this result is reproducible, then the optimizing approach used has reduced mortality from secondary disease to a 90-day mortality level quite similar to that seen with isologous bone marrow therapy, where secondary disease is not a factor. Further experiments will be needed to determine the levels of these four variables that cause greatest 90-day mortality from secondary disease.

References

[1]In collaboration with M. A. Kastenbaum and D. A. Gardiner, Biometrics Group.

8.2 OXYGEN CONSUMPTION IN RADIATION CHIMERAS

W. H. McArthur[1]

Introduction. – Secondary disease is a disorder that frequently kills irradiated mice transplanted with foreign bone marrow. It has been proposed that the disease represents a metabolic disturbance initiated by the graft against host immune reaction.[2]

Studies by McArthur *et al.* showed that mice given foreign bone marrow after lethal irradiation

stayed in positive nitrogen balance for 90 days, even in the presence of a small weight loss. A very mild clinical secondary disease was present in these mice.

As a continuation of whole-animal metabolism studies in radiation chimeras, the present report summarizes oxygen consumption in lethally irradiated mice given foreign bone marrow. These measurements were made over a period of 90 days after irradiation and bone marrow transplantation. Measurements on normal and isologous animals extended through day 305.

Materials and Methods. – Three groups of animals were studied. One group was a normal control, and two groups received 900 r of total-body x irradiation followed within 1 hr by an intravenous injection of 40×10^6 bone marrow cells of isologous (IBM) (ref. 3) or homologous (HBM) (ref. 4) origin. Oxygen consumption measurements were made several times a day on animals in each of the three groups; 12 normal, 10 IBM, and 17 HBM animals were used in this study. All HBM mice were dead by the end of the 13th week after irradiation. Linear regression lines were fitted to the data to obtain least-squares estimates of the slopes and intercepts. Standard errors were obtained to test for significance of differences in oxygen uptake in the three groups.

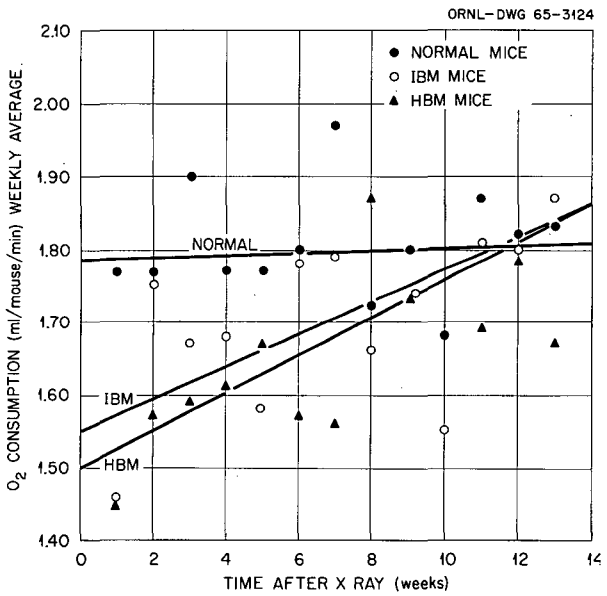

Fig. 8.2.1. Average Oxygen Consumption Measurements in Normal, IBM, and HBM Mice at Weekly Intervals After Start of the Experiment. The linear regression lines for each of the three groups are shown.

Results. – Figure 8.2.1 shows that normal animals used significantly more oxygen than IBM or HBM animals during a 13-week period. The IBM and HBM groups were not different from one another according to the statistical tests. The response of the IBM group over the entire 44-week period was not linear, and separate regression lines were plotted for the IBM and normal mice from 14 to 44 weeks. During the 14- to 44-week interval, the oxygen uptake of the IBM group remained significantly lower than that of the normal mice. These results indicate that the decrease in oxygen uptake is associated with radiation damage rather than with foreign bone marrow transplantation.

References

[1]Consultant from Knoxville College, Knoxville, Tenn.

[2]A. L. Kretchmar and C. C Congdon, *Am. J. Physiol.* **200**, 102 (1961).

[3]$(C_{57} \times A/H_E \, ♂)F_1$ J or Cum.

[4]$(101 \times C_3H)F_1$ Cum.

8.3 CHANGE IN THE PARTITION OF TOTAL-BODY NITROGEN IN IRRADIATION CHIMERAS

A. L. Kretchmar[1]

Introduction. – We have previously reported[2] that nitrogen balance in irradiated mice given homologous bone marrow cells was positive and that these animals retained as much nitrogen as did irradiated mice given isologous cells. Despite this equally favorable nitrogen balance, the homologous chimeras failed to gain weight, whereas mice given isologous cells showed good weight recovery and growth. Both irradiated bone-marrow-treated groups retained more nitrogen over the 90 days following irradiation than normal unirradiated control mice.

It was tentatively concluded that the nitrogen retained by irradiated mice treated with isologous cells was utilized in synthesis of protein for the regenerating hemopoietic system, other repair

processes, and growth. We speculated that since the homologous chimeras retained as much nitrogen but failed to gain in body weight, there was a shift in the distribution of body nitrogen in these mice with homologous marrow grafts. If, for example, nitrogen were bound up in the protein of antigen-antibody complexes of a graft-vs-host reaction and these complexes were deposited extracellularly or on the surface of cells of the graft and host,[3] then a considerable amount of nitrogen could be retained without increasing the body weight to the same degree as if this same amount of nitrogen were retained in protein of normal protoplasm, where there is approximately 4 g of water for each gram of protein. Further, if the formation of antibody occurred at a rate high enough to mobilize body protein, then the shifting of nitrogen from normal tissue compartments to extracellular antigen-antibody complexes could result in loss in gross body weight not reflected in nitrogen balance studies, where only nitrogen intake and excretion are measured.

Results and Discussion. – To test this suggestion, the total-body nitrogen of mice was determined 4 and 25 days after lethal x irradiation and injection of isologous or homologous bone marrow cells (40×10^6). The animals were arbitrarily divided into four sections by gross dissection: I, blood and thoracic viscera; II, abdominal viscera; III, skin, tail, and head; and IV, carcass. These sections were completely digested in sulfuric acid, and the nitrogen content was determined. The total-body nitrogen was thus partitioned into four compartments.

The results are summarized in Table 8.3.1. The numbers shown were calculated by dividing the total nitrogen content of section III by that of section IV. When the ratios for each animal were plotted together against body weight, there was no correlation in normal and isologous-marrow-treated animals. This showed that the total content in each section varied together with body weight (thus, small animals had less nitrogen in both skin and carcass), but that the relative amount of the total-body nitrogen in skin or carcass was not different in 24-g animals from that in 14-g animals (14–24 g was the weight range of animals in the normal group). In contrast, in homologous chimeras the skin/carcass ratio was negatively correlated to body weight. That is, the lightest chimeras had the highest ratios.

Table 8.3.1. Ratio of Nitrogen in Skin to That in Carcass of Mice Given 950 r of Total-Body Irradiation and 40×10^6 Bone Marrow Cells

Group and Marrow Treatment	Days After Irradiation	Skin/Carcass Nitrogen Ratio
Normal	(4-day control)	1.0
	(25-day control)	1.0
Isologous	4	1.0
	25	1.0
Homologous	4	1.24[a]
	25	1.43[a]

[a]Ratios are for chimeras with body weight less than 18 g. Animals above this weight showed normal skin/carcass ratios.

The data in Table 8.3.1 show that distribution of nitrogen is changed in homologous chimeras and that there is relatively more nitrogen in skin than in carcass, especially in animals weighing less than 18 g and, therefore, presumably suffering from homologous disease. The data also show that this shift can be detected early (four days) but is probably progressive. The result suggests that a major target of the graft-antihost reaction is the skin; and that nitrogen is deposited in this tissue at the expense of the carcass tissues.

References

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8.4 ELECTRON MICROSCOPIC OBSERVATIONS ON SPLENIC WHITE PULP DURING THE EARLY INTERVALS OF A PRIMARY IMMUNE RESPONSE

D. C. Swartzendruber

Introduction. – The definitive ultrastructural observations have not yet been made on the precursors of plasma cells, due mainly to the inability to recognize the earliest members of the family of cells responding to antigenic stimulation. The present study was undertaken to systematically describe the morphologic aspects of hemocytoblasts which appear in the white pulp of the mouse spleen during the first three days of a primary response to chicken erythrocytes.

Results and Discussion. – An increase in the number of large blast cells with deeply basophilic cytoplasm occurs in mouse splenic white pulp within 24 hr after an intravenous injection of foreign erythrocytes. These large cells are characterized ultrastructurally by many freely dispersed ribosomes, although clusters and spiral configurations are observed. A large Golgi zone is present, along with swollen-appearing mitochondria, many with incomplete cristae. Large, generally irregularly shaped nuclei with hypertrophied nucleoli containing dense nucleonema further characterize these cells. Three types of cells are distinguished among the increased number of blast cells present in white pulp two days after antigen injection. A highly developed endoplasmic reticulum characterizes one cell type. A second type of cell contains a uniformly round nucleus, a well-developed Golgi region, and shows no development of endoplasmic reticulum. A third cell type located in white pulp sinuses contains many clusters of ribosomes, a large Golgi region, and only a few profiles of endoplasmic reticulum. The periphery of the while pulp contains layers of immature plasma cells at day 3. Only a few large cells of the second type described above were observed in white pulp at day 3. It is suggested that both plasmacytopoiesis and lymphocytopoiesis occur simultaneously in white pulp during the early intervals of a primary immune response.

8.5 EFFECT OF INCUBATION TEMPERATURE ON SURVIVAL OF IRRADIATED MOUSE BONE MARROW CELLS

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

Introduction. – We have been investigating a system whereby effects of in vitro postradiation environment on transplantation and proliferation of mouse bone marrow cells could be studied quantitatively. In previous reports,[1] culture conditions were described which gave optimal retention of normal marrow activity after 6 hr in vitro. The spleen ^{59}Fe method used to assess marrow activity has been reported.[2] The present report deals with the effect of postirradiation temperature on erythropoietic activity of irradiated marrow suspensions.

Methods. – Femoral marrow was removed from normal donor mice and suspended in TC 199 containing 25% calf serum and penicillin-streptomycin. Cell concentration was adjusted to 5.0×10^6/ml, and the suspension was divided into two aliquots of ~10.0 ml. One aliquot was exposed to 100 or 150 r of 250-kv x rays. Both aliquots were incubated for up to 24 hr at 2, 10, 18, 22, 25, or 37°C. After incubation, suspensions were diluted with Tyrode's solution so that each milliliter contained 0.5×10^6 cells. The suspensions were tested for erythropoietic activity as previously described.[1]

Results and Conclusions. – Table 8.5.1 shows results of 11 separate experiments in which erythropoietic activity (^{59}Fe counts/min/spleen) of irradiated cells was compared with that of unirradiated cells at different culture temperatures. In making the calculation $A/B \times 100$, it was assumed that loss of activity due solely to incubation was the same in both irradiated and unirradiated cultures. Previous results showed this loss to be temperature dependent. Counts in column B represent the potential of unirradiated bone marrow at 2, 6, and 24 hr of incubation, and the difference in counts (B minus A) at any one incubation temperature represents effect of in vitro irradiation. Surviving cells can be calculated as $A/B \times 100$. Results indicate that the percent of cells surviving 100 or 150 r increased

Table 8.5.1. Effect of Postirradiation Culture Temperature on Erythropoietic Activity of X-Irradiated Mouse Bone Marrow Cells

Experiment Number	Incubation Time (hr)	Incubation Temperature (°C)	In Vitro X-Ray Dose (r)	Spleen ^{59}Fe (counts/min/spleen)[a]		Surviving Cells ($A/B \times 100$)
				Irradiated (A)	Unirradiated (B)	
1	2	37	150	1068	4966	21.5
2	2	22	150	1894	4543	41.7
3	2	2	150	2698	4877	55.3
4	6	37	150	352	2684	13.1
5	6	22	150	780	3356	23.2
6	6	2	150	1001	3113	32.2
7	24	37	100	53	708	7.5
8	24	22	100	1342	4670	28.7
9	24	18	100	1132	4036	28.0
10	24	10	100	2015	4216	47.8
11	24	2	100	2044	3879	52.7

[a]Each value in columns A and B represents the average of 5 to 7 mouse spleens.

as the incubation temperature decreased. This effect was observed at 2, 6, and 24 hr of incubation. It appears that holding marrow cells at a lower metabolic state reduced the detrimental effects of irradiation, although the basis of this reduction is not yet clear. Similar results were reported by Beer *et al.* for cultured mouse leukemia cells.[3] Whether the postradiation temperature effect is a true repair remains to be shown.

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8.6 PERSISTENCE OF P1 SPLEEN CELLS INJECTED INTO ADULT P2 MICE MADE TOLERANT AT BIRTH BY INJECTION OF (P1 × P2)F_1 CELLS

Joan Wright Goodman

Introduction. – Aside from rejections based on sex-related antigens, parental skin grafts are never rejected from their F_1 hybrids. This has been interpreted to mean that all antigens of both parents are present in the hybrid cross. Data inconsistent with the so-called laws of transplantation, based in part on this interpretation of skin graft data, have appeared in the literature. Some of these data, showing poor growth of parental (P1) cells in irradiated (P1 × P2)F_1 hybrids (F_1), have been interpreted to mean that the parental cells contain an antigen or antigens not found in the hybrid and that the hybrid therefore can and does react immunologically against its own parent.[1,2] Should such antigens actually

exist, the published exceptions to transplantation laws being open to additional interpretations, these antigens should be demonstrable by a variety of techniques, including the one reported here.

Methods. – Parental mice (P1) were injected intraperitoneally within 12 hr of birth with approximately 2×10^7 spleen cells from F_1 hybrid males. Some of the mice, at three to nine months of age, were tested for tolerance to the second parental strain (P2) by skin grafts. They were considered highly tolerant if the P2 graft remained in good condition for more than 100 days. Some of these highly tolerant mice and others injected at birth but not tested as adults with skin grafts were tested for tolerance to a suspension of P2 spleen cells (10^8) inoculated intraperitoneally. These mice, along with appropriate controls, were observed for possible weight loss (indicative of the development of wasting disease), and after a period of time their lymph node cells (LNC), bone marrow cells (BMC), and peritoneal fluid cells (PFC) were examined cytotoxically (by reaction with P1- and P2-specific isoimmune sera in the presence of rabbit complement) for the presence of donor cells.

Parental strains and their F_1 hybrids used were C57BL/6 Cum (hereafter called B6), DBA/2 Cum (D2), [B6♀ × D2♂]F_1 (B6D2F_1), C57BL/Cum (B), C3H/Anf Cum (C3), and [B♀ × C3♂]F_1 (BC3F_1).

Results and Discussion. – Many (49 out of 76) of the P2 spleen-inoculated mice lost weight and showed histologic evidence of wasting disease in lymphatic tissues. Twenty-eight of these mice showed only transient losses, whereas the remaining 21 showed progressive loss (up to 38% within 3–5 weeks) to the point of death or near death, at which time they were killed for further study. Among the 27 similarly treated mice that did not show any evidence of secondary disease were 13 carrying P2 skin grafts.

At first, only lymph node and bone marrow cells were analyzed cytotoxically, and these studies were done only on P1 mice that had lost weight (most of them more than 20%), usually 3 to 5 weeks following inoculation of P2 spleen cells. The first 29 mice showed no evidence of donor-type cells in the tissues studied.

It is possible that the P2 cells had actually persisted and engaged in an active graft-vs-host reaction but had not been found because they were looked for too late, after they had passed the peak of proliferation and activity. For this reason, the next groups of mice were analyzed cytotoxically at 14 days after receiving P2 spleen cells whether they had lost weight or not. Peritoneal fluid cells were typed, and, if they gave evidence of chimerism, lymph node and marrow cells from that mouse were also typed. These data are shown in Table 8.6.1. All chimeric mice were of C3 or D2 strains; no tolerant B's or B6's were found. This is the same result observed with skin grafts.[3] It can be seen that about half the mice tested relatively early after receiving spleen cells contained donor cells in the peritoneal cavity. Of these, about half also showed evidence of donor cells in lymph nodes. Other lymph nodes taken from these mice appeared histologically to be very reactive. However, no marrow chimerism was found in any of the tolerant P1's.

Conclusion. – These results show that tolerance to all antigens present in P2 spleen cells can be induced in P1 mice by the neonatal injection of (P1 × P2)F_1 spleen cells. The inductively

Table 8.6.1. Summary of Cytotoxicity Data of Tolerant P1 Mice

Neonatally Injected (with F_1) P1 Mice, Injected as Adults with P2 Spleen	Number Showing >20% Weight Loss When Analysis Done	Number Showing Chimerism/Number Tested		
		PFC	LNC	BMC
Not skin grafted	18/24	0/0	0/24	0/24
Bearing P2 skin grafts	3/21[a]	10/21	6/10	0/10

[a]Most of these sampled at 14 days after P2 spleen cells.

tolerant P1 does not differ in this respect from the naturally or genetically tolerant F_1, which as an unirradiated adult also tolerates P2 spleen cells and develops chimerism and wasting disease as a result of P2-anti-F1 immunologic activity.

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8.7 A NEW IN VIVO CULTURE TECHNIQUE FOR MAMMALIAN CELLS

Henrianne G. Willard

Introduction. – Under ideal conditions, mammalian cells placed in tissue culture should proliferate and maintain their normal functional activity. Although some progress has been made in this direction, most mammalian tissues quickly lose both their morphologic and functional identity in culture with standard in vitro techniques. Development of the intraperitoneal diffusion chamber in vivo culture method offers some advantages in cell maintenance, but the technique requires considerable skill in operation; the total volume of the chamber is quite small (0.2 ml); and many of the cells clump together and are not harvestable. It was found in the present work that dialysis tubing can be used as a convenient culture chamber that will maintain cells in reasonably good condition for short-term culture in the peritoneal cavity.

Materials and Methods. – Six-inch lengths of dialysis tubing ($^{20}/_{32}$ in. in diameter) were opened, tied off tightly at one end, autoclaved in saline, and refrigerated. Bags were removed from saline, drained, and filled with 2 ml of culture medium (generally TC 199 with added serum) containing mouse hematopoietic cells at the desired concentration. The open end of the bag was tied securely before being implanted in the peritoneal cavity of a mouse for in vivo culture. At termination of the culture period, the bag was removed and restored to original volume. Cells could then be examined for morphologic appearance and functional activity.

Results and Discussion. – Parallel in vitro and in vivo cultures of mouse spleen were analyzed for immunological competency.[1] After two days of culture, spleen cells from the dialysis bag were approximately 1.3 times as active as cells grown in vitro. After seven days of culture, cells from the dialysis bag were only slightly better than cells from the in vitro culture.

Mouse bone marrow or spleen cells were cultured in dialysis bags in mice or in guinea pigs. At daily intervals after initiation of culture, cells were harvested and were injected into isologous, lethally irradiated mice. Both spleen cells (20×10^6) and marrow cells (2×10^6) were able to promote 30-day survival up to three days of in vivo culture. After four days of culture, these cells were ineffective at the two doses tested. Samples of cultured cells were smeared and stained for microscopic observation. Many intact nucleated cells were found at early time intervals (up to a week), and fewer, but still intact, cells were found up to three weeks of in vivo culture. Longer culture intervals were not studied.

Other applications of the dialysis bag culture technique are being explored. Human leukocytes can be cultured successfully in the presence of phytohemagglutinin (PHA) in dialysis bags in the peritoneal cavity of a mouse, and some mitotic figures can be obtained from human leukocytes in the absence of PHA. Also, guinea pig marrow and leukocytes can be successfully cultured for short periods within the peritoneal cavity of isologous guinea pigs. The possibility of culture of cells in vitro in dialysis tubing with an appropriate circulating external medium is also being considered.

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8.8 BACTERIOLOGICAL STUDIES ON IRRADIATED MICE INOCULATED WITH A PIGMENTED AND A NONPIGMENTED *PSEUDOMONAS*

M. L. Simmons J. A. Franklin

Introduction. – Routine object sampling procedures in the Experimental Animal Facility have

shown a high incidence of a nonpigmented *Pseudomonas* (*P. stutzeri*). We set out to determine if this organism is involved in the early death syndrome in x-irradiated mice by comparing the pathogenicity of *Pseudomonas aeruginosa* with that of *Pseudomonas stutzeri* on the basis of time of death and subsequent recovery of the organism(s) from irradiated animals. Various investigators have reported changes in pigmentation in *P. aeruginosa* in culture. We wanted to determine if this phenomenon could be brought about in vivo after irradiation of the host animal, using *P. aeruginosa*.

Materials and Methods. – Animals used were adult specific-pathogen-free (SPF) $C3BF_1$ (C3H/Anf Cum ♀ × C57BL/Cum ♂) mice 10 to 14 weeks of age supplied by Cumberland View Farms. All animals were randomized within each sex and placed five animals per pan. Water containing 10 to 14 ppm free chlorine was placed on the cages, and the water bottles were changed three times per week throughout the experiment. Fecal samples from each pan of mice were tested bacteriologically for the presence of *Salmonella* and *Pseudomonas* on two consecutive weeks before the animals were to be used in the experiment. Neither of the pathogens was found. All irradiated animals received 950 r of 250-kv x rays.

Twenty-five animals were infected approximately 2 hr before irradiation with 1×10^6 *P. aeruginosa* organisms per mouse by intraperitoneal injection. Twenty-five animals were infected with the same dose by oral lavage 24 hr before irradiation. Ten control animals were infected in the same manner and with the same number of organisms, but were not subjected to x rays. Ten additional animals were held with the infected mice but were not given *Pseudomonas* or subjected to x rays. Identical inoculation and control procedures were used with those mice infected with *P. stutzeri*, except that they were given 1×10^7 organisms per mouse. Gaby and Logan[1] found that some nonpigmented strains of *P. aeruginosa* were as lethal to mice as the pigmented strains if given in increased numbers, but that generally the blue-green pigmented strains were more pathogenic for mice than the other pigmented or nonpigmented varities.

Culture Methods. – The presence of *Pseudomonas*, pigmented or nonpigmented, in the blood stream was determined by culturing the heart blood clots of animals dying during ten days

Table 8.8.1. Mortality and *Pseudomonas* Recovery in Animals Infected with Pigmented and Nonpigmented *Pseudomonas*

Mouse Death (day)	Intraperitoneal *Pseudomonas* – 25 Animals		Oral *Pseudomonas* – 25 Animals		Controls – 10 Animals		No *Pseudomonas*, No X Ray – 10 Animals
	Number Dying	Number with *Pseudomonas*	Number Dying	Number with *Pseudomonas*	Intraperitoneal *Pseudomonas*, No X Ray	Oral *Pseudomonas*, No X Ray	
1	0	0	0	0	0	0	0
2	2	2	0	0	0	0	0
3	2	2	0	0	0	0	0
4	3	3	(1) 0	(0) 0	0	0	0
5	5	3	2	2	0	0	0
6	8	8	3	3	0	0	0
7	3	2	2	2	1[a]	0	0
8	1	1	(1) 1	(0) 1	0	0	0
9	(2) 1	(0) 0	(1) 2	(0) 1	0	0	0
10[b]	(6) c	(0)	(5) 5	(0) 1	0	0	0

[a]Pigmented *Pseudomonas* recovered.

[b]None of the surviving irradiated or control mice killed on day 11 showed *Pseudomonas*.

[c]All animals were dead before the 10th day.

( ) Indicates animals dying in the parallel group that received nonpigmented *Pseudomonas*.

postirradiation. At the end of ten days all controls and the surviving irradiated animals were killed, and cultures were taken of the heart blood and minced intestines. The inocula were placed in thioglycolate broth (Difco) and incubated at 37°C for 48 hr. At the end of the incubation period the broth culture was streaked on glycerol or "Tech" agar plates[2] and incubated at 25°C for 48 hr. After the incubation period the plates were examined for the presence of pyocyanin production. Those cultures not showing pigmentation were then tested for the presence of cytochrome oxidase for the detection of nonpigmented *Pseudomonas*.[3]

Results and Conclusions. – In comparing *P. aeruginosa* and *P. stutzeri* by animal deaths and subsequent recovery of the organism(s), Table 8.8.1 shows that nonpigmented *Pseudomonas* did not produce lethal effects on $C3BF_1$ mice. Under the conditions outlined in this study, this organism does not seem to be involved in the early-death syndrome. Also, because of delayed death time and failure to recover any pigmented *Pseudomonas* from those mice infected with *P. stutzeri*, it is assumed that this organism does not revert to a pigmented variety in vivo.

Conversely, nonpigmented *Pseudomonas* organisms were not recovered from inocula of those animals infected with pigmented *P. aeruginosa*. Under defined conditions of this study, this seems to preclude the possibility of this organism reverting or dissociating to a nonpigmented variety in vivo.

In comparing routes of infection by *P. aeruginosa*, the intraperitoneal route resulted in higher mortality and greater subsequent recovery of organisms. The lower mortality and recovery following the oral lavage route of infection could be due to elimination of the organisms through the intestional tract,[4] as well as the bactericidal action of chlorine-treated water.

An unidentified nonhemolytic *Streptococcus* was present in cultures from those animals dying on days 9 and 10 after injection with *P. aeruginosa*. This *Streptococcus* was also the predominant organism present in cultures from the heart blood clot inocula of those animals receiving x radiation and *P. stutzeri* injections. This organism was not observed in cultures of heart blood inocula from control animals. Culturally, this organism does not resemble fecal *Streptococcus*.

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9. AEC-NASA Space Biology Program

Space Biology – High-Energy Proton Studies (9.1, 9.2)

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Space Biology – Biosatellite (9.3, 9.4)

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Space Biology – Gemini III (9.5)

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9.1 EFFECTIVENESS OF HIGH-ENERGY PROTONS AND OTHER FAST CHARGED PARTICLES ON VARIOUS BIOLOGICAL MATERIALS

Cellular Systems

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This portion of the Biology Space Program is no longer funded by NASA. However, the final report is being prepared and includes data from experiments other than those which involved irradiation of materials with primary beams of protons and heavy ions. For example, comparative data from neutron irradiations are included.

Most of the results were reported in our NASA-AEC Space Biology Annual Report, July 1964, ORNL-TM-924.

Analysis of the data shows that the maximum RBE has not been measured for inactivation or mutation in *Neurospora*, lethality of mouse spermatogonia and oocytes, or for production of chromosomal aberrations in human leukocytes. It is important to note that RBE's between 5 and 9 have been obtained in these systems with the highest-LET radiations used.

For estimations of hazards due to high-LET radiations, it would be advantageous to have measurements of the maximum RBE for several systems, especially for mammalian and human cells.

9.2 STUDIES ON THE DELAYED SOMATIC EFFECTS OF PROTON IRRADIATION IN MAMMALS IN THE OAK RIDGE ISOCHRONOUS CYCLOTRON: IRRADIATION OF THE MOUSE LENS WITH 35-Mev PROTONS

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K. W. Christenberry

An air-conditioned trailer for housing and transportation of animals to distant accelerator sites has been received, tested, and placed in use. With this trailer and with the completion of the special biology facility at the Oak Ridge Isochronous Cyclotron,[1] we have now initiated the irradiation of mice with fast charged particles, as planned.[2]

A study of the effects of 35-Mev proton irradiation of the mouse lens was undertaken as our first experiment, pending the availability of protons of (60–70)-Mev energy. Existing data,[2] moreover, indicated the need for investigating the cataractogenic effectiveness of radiation comparable in LET to 35-Mev protons.

In the 35-Mev experiment, 175 10-week-old female RF mice were exposed to single absorbed doses of 50–1000 rads. Protons at a dose rate of about 10–40 rads/sec were delivered to one eye of each animal, the other eye serving as a control. The particle energy and conditions of irradiation were chosen so that the lens was exposed on the relatively flat, low-LET part of the ionization curve rather than on the Bragg peak. Comparable groups of animals were irradiated with corresponding amounts of 300-kev x rays delivered to the head, with the rest of the body shielded. The eyes of the mice are being examined periodically with a slit lamp for lens changes and other effects. Initial results suggest that 35-Mev protons are appreciably more effective than 300-kev x rays for producing radiation cataracts, with easily detectable opacities of the lens observable by two and one-half months after exposure to 200 rads of protons. Precise estimation of the RBE for lens injury must, however, await further data.

Experiments with (60–70)-Mev whole-body proton radiation are scheduled to begin in the immediate future, as planned.[2]

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9.3 BIOSATELLITE A PREPARATORY EXPERIMENTS

F. J. de Serres R. C. von Borstel
B. B. Webber Anna R. Whiting

A number of preparatory experiments are now in progress to establish the possible effects of dose rate, temperature, dehydration, anoxia, and other parameters imposed on the biological test materials by the nature of the proposed Biosatellite experiment. The actual mockup experiments are anticipated during the reporting period.

The progress reports of the individual investigators appear in this report in the appropriate sections: (1) Cytochemistry and Cell Reproduction – R. C. von Borstel and Anna R. Whiting; (2) Cytology and Genetics – F. J. de Serres and B. B. Webber.

9.4 SPONTANEOUS MUTATION FREQUENCIES IN *HABROBRACON*

A. R. Whiting Diane J. Goins
R. C. von Borstel Martha J. Bond

Introduction. – In the course of testing sex alleles of various wild-type strains of *Habrobracon*, it was found that the Raleigh wild-type (R) was especially vigorous. This stock has sex alleles different from the wild-type stock No. 33, so that crosses between them give no diploid males. Diploid males are characterized by high inviability and sterility. The lemon mutant strain (with sex alleles derived from stock No. 33), a mutant readily identifiable in the heterozygous condition as well as in the pure stock, was used as the source of males for general experimentation. The first experiment using these two strains (R and lemon) was that of determination of the spontaneous mutation frequency.

Results and Discussion. – Raleigh females were isolated, tested for presence of lethals, and then mated to lemon males, after which tests for presence of lethals were continued. F_1 females from lethal-free parents were set unmated, and individual records for offspring (haploid) viability were principal criteria for the 767 females tested and their 18,785 eggs. Of these females, 6 died early in the experiment, and 10 could be listed as sterile females. Out of the 751 remaining, 6 females (0.79%) were classed as being heterozygous for "semilethals" or oogonial lethals, since the hatchability of their eggs was between 60% and 80%. Two females out of 751 (0.26%) were considered to be heterozygous for spontaneous recessive lethals, since the hatchability of their eggs was below 60% and above 0. The remainder were lethal free. One female produced 20 offspring, 2 of which were white eyed. We believe that this visible mutation originated in an oogonial cell of the mother, since the mutant and wild-type sons were not found in equal numbers.

The results of this experiment demonstrate the reliability of these stocks and serve as background information for future use in radiation synergism studies.

9.5 GEMINI III EXPERIMENT

M. A Bender P. Carolyn Gooch

The progress in this experiment has just been reported in the annual report, *Synergistic Effect of Zero-G and Radiation on White Blood Cells. An Experiment for the Gemini III Manned Space Flight,* ORNL-TM-940 (January 1965). The report contains a complete coverage of development and testing of the actual package to be used.

10. Radiation Immunology

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10.1 SUPPRESSIVE EFFECT OF SUPRAOPTIMUM DOSES OF ANTIGEN ON THE SECONDARY ANTIBODY-FORMING RESPONSE OF SPLEEN CELLS CULTURED IN CELL-IMPERMEABLE DIFFUSION CHAMBERS

Takashi Makinodan Immo Hoppe
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Introduction. – Previous studies have shown that suboptimum secondary immune response can be easily induced by culturing spleen cells from adequately primed donors with a supraoptimum dose of either particulate or soluble antigen (100 to 1000 × optimum dose).[1,2] Subsequent studies were carried out in an attempt to understand the cause of this suppression.

Methods and Results. – A fixed number of spleen cells from adequately primed donors were cultured in diffusion chambers with suboptimum, optimum, and supraoptimum doses of sheep red blood cells or bovine serum albumin (BSA). Autoradiographic and immunofluorescent methods were employed in detecting antibody-forming cells. Autoradiographic studies with the use of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-proline revealed that the decrease in T_2 of the lymphoid cell population after stimulation of spleen cells with an optimum dose of antigen is due to preferential proliferation of metabolically active blast cells and plasma cells, and the decrease in antibody output caused by suboptimum and supraoptimum doses of antigen can be related mainly to the number of metabolically active plasma cells. Immunofluorescent studies revealed that, when primed spleen cells were cultured with a supraoptimum dose of BSA, the number of functional antibody-forming cells decreased markedly, and, in addition, the efficiency of the functional cells seemed to be reduced to one-fourth the normal.

Discussion. – In regard to the transformation of progenitor cells (PC_1 and PC_2) into functional antibody-forming cells (P), it was not known whether the deleterious effect of the antigen in tolerance induction was on the primary transformation event between PC_1 and PC_2, on the secondary transformation event between PC_2 and P, or on both. The results presented here indicate that the antigen affects at least the PC_2-to-P transformation event. They also show that, when stimulated with a supraoptimum dose of antigen,

progenitor cells are not differentiating and proliferating into functional antibody-forming cells. Whether this is due to death or metabolic paralysis is not known.

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10.2 VARIATION IN THE KINETIC INDICES OF PLASMA CELLS IN ANTIBODY RESPONSE

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Introduction. – Two of the major unsolved problems in the field of cytodynamics of antibody formation are (1) the fate of terminal plasma cells and (2) the intercompartmental transit time, or the time it takes for a cell to transform from one metabolically unique state into another (ITT). The present study was carried out in an attempt to resolve these problems.

Materials and Results. – A fixed number of primed spleen cells (2.4×10^7) were cultured in 0.1-μ porosity diffusion chambers, together with an optimum dose of antigen ($\sim$50 μg of bovine serum albumin). Autoradiographic and immunofluorescent methods were employed in detecting antibody-forming plasma cells. The results showed that mature antibody-forming cells in the stationary phase are derived from proliferating immature plasma cells in the late latent and log phases of response and that 70% of all plasma cells at this time are immunofluorescent-positive antibody-containing cells. Furthermore, the evidence indicated that the life cycle of immature plasma cells was changing during the log-stationary phase of response, and, associated with the change in the life cycle of immature plasma cells, the ITT between immature and mature plasma cells was varying from 3 to 12 hr. Finally, mature plasma cells were shown to be dying exponentially, with a $T_{1/2}$ of approximately 32 hr.

Discussion. – These results indicate that, because the ITT can vary from 3 to 12 hr, there must exist many "transitional" plasma cells that possess properties between those of typical immature and those of mature plasma cells. Such cells have been observed by morphologists. The data also show that at any given time a newly formed mature plasma cell has a probability of dying equal to that of one which was formed much earlier. It follows then that senescence in the classical sense cannot be the primary cause for death of mature plasma cells.

10.3 ANTIGEN COMPETITION AS AN INDEX OF DIFFERENTIATIVE STATUS OF IMMUNOLOGICALLY COMPETENT CELLS

J. F. Albright T. W. Evans

Introduction. – It is a reasonable presumption that analysis of competition of noncross-reacting antigens might provide information concerning the number and differentiative status of cells in which antibody production is initiated. A number of studies of competition of antigens have been reported, but they are generally inadequate as a result of failure to consider antigen dosages, the proliferative cycle of competent cells, and other variable factors. As a result of our interest in and evidence for pluripotentiality of competent cells, it is imperative to obtain more precise information about antigen competition.

Methods and Results. – Twelve-week-old $C31F_1$ mice were employed in these studies. Three noncross-reacting erythrocyte antigens were used: rat erythrocytes (Ag_1), sheep erythrocytes (Ag_2), and chicken erythrocytes (Ag_3). It was reported previously[1] that maximum competition was observed when the injection of Ag_2 preceded the injection of Ag_1 by a period of 12 to 24 hr. Our principal aim in the studies reported here was to ascertain the extent to which the response to Ag_1 could be obliterated by manipulating the number of antigens employed and their relative dosages. The procedure was to inject normal mice with one or both of the competing antigens (Ag_2 and Ag_3) at various intervals prior to injection of a standard test dose of the antigen (Ag_1), which was used for assessing residual capacity for antibody response.

Table 10.3.1. Competition of Noncross-Reacting Antigens Administered Under Various Conditions

Number of Crbc[a]	t^b (hr)	Number of Srbc[a]	t^b (hr)	Number of Rrbc[a]	Percent Antibody Produced Relative to Normal Primary Response	
					Rat	Sheep
		2×10^9	8	2×10^8	9	100
		2×10^9	16	2×10^8	12	100
		2×10^9	16	4×10^9	39	
		6×10^9	16	4×10^9 + (2×10^9 Srbc)	41	
2×10^9	15	2×10^9	11	2×10^8	5	13
10^9	2	6×10^9	10	4×10^9	36	100

[a]Crbc = chicken erythrocytes; Srbc = sheep erythrocytes; Rrbc = rat erythrocytes.
[b]t = interval between administration of two antigens.

Some of the results are shown in Table 10.3.1. The amount of Ag_1 sufficient to give a maximum antibody response in the normal animal is 2×10^8 cells. Yet, when preceded by Ag_2 or Ag_2 and Ag_3, the same dosage of Ag_1 elicits production of only 5 to 10% of the normal amount of antibody. This suggests competition of antigens for pluripotential competent cells. However, under the same experimental conditions, increasing the dose of Ag_1 by a factor of 20 results in antibody response which is 35 to 40% of normal. None of the conditions employed thus far have enabled us to further reduce this response to the high dose of Ag_1.

Discussion. – Our data support the idea that there exist immunological progenitor cells which are pluripotential with regard to antibody production to different antigens. The data also suggest the existence of other cells which are unaffected by prospective competing antigens and might be considered monopotential. However, the data that suggest the existence of monopotential progenitors may be explained in other ways, for example: (1) the antigens are administered intravenously, and the first antigen to reach the spleen in large amount in a short interval of time might "physiologically splenectomize" the animal with respect to a second antigen, and (2) high doses of test antigen might force precocious differentiation of pluripotential progenitor cells under conditions where progenitors are in short supply.

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10.4 RECOVERY OF THE SECONDARY ANTIBODY-FORMING POTENTIAL OF SPLEEN CELLS AFTER X IRRADIATION

Paul Nettesheim

Introduction. – Previous study in this laboratory showed that the antibody-forming potential of primed spleen cells is as radiosensitive as that of nonprimed spleen cells.[1] However, information on its recovery pattern after x irradiation is lacking. Studies were therefore carried out to obtain such information after exposure of primed spleen cells to different doses of x irradiation.

Methods and Results. – $(C3H \times 101)F_1$ mice primed at eight weeks of age with an optimum

dose of rat red blood cells were irradiated four weeks later with 0, 200, 400, or 600 r. At varying intervals thereafter, their spleens were removed and cell suspensions were prepared. A fixed number of cells were then injected intraperitoneally, together with an optimum dose of the test antigen, into lethally irradiated isologous mice, which served as the "culture test tubes." Our preliminary experiments showed that the stationary phase of response was attained eight days after culture, and, therefore, eight-day response studies were carried out subsequently. The antibody-forming potential of primed spleen cells immediately after x-ray treatment was reduced to 35, 6, and 0.4% of the original level when the cells were exposed to 200, 400, and 600 r respectively. During the first six to ten weeks after irradiation, the activity per fixed number of spleen cells and per spleen equivalent increased by no more than two- to threefold in all three radiation groups.

Discussion. – These results indicate that recovery of the secondary antibody-forming potential of primed spleen cells is minimal during the first two months after exposure to 200 to 600 r. On the other hand, previous investigators reported that the secondary antibody-forming potential of intact animals recovers rapidly after x-ray treatment. In view of these results, further studies are now under way in an attempt to understand the role of the primary antigen dose in relation to the time interval between the primary antigen injection and the x irradiation on recovery pattern of the secondary antibody-forming potential of spleen cells and intact mice.

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10.5 RADIORESISTANT NATURE OF PHAGOCYTIC CELLS

E. H. Perkins Toshiteru Morita

Introduction. – The problem of the relative radiosensitivity of phagocytic cells has never been resolved. This is due in part to the lack of suitable assay methods. The majority of the past radiation studies were carried out in situ and therefore were limited to use of only nonlethal doses of x irradiation. The phagocytic activity of individuals was generally assessed indirectly by determining the rate at which target particles disappeared from the circulation or deposited in the various organs. In this study the effect of high levels of x irradiation on phagocytic cells was assessed kinetically using an in vitro culture method.

Methods and Results. – Mouse peritoneal phagocytes were irradiated and cultured in vitro in 25-ml Erlenmeyer flasks (3 ml containing 2×10^7 phagocytes/ml). An equal volume of opsonized sheep erythrocytes (RBC) (4×10^7/ml) was added 0, 6, 12, or 24 hr later. Free RBC, engulfed RBC, and the supernatant were analyzed for their hemoglobin content at intervals after addition of target RBC. This permitted us to assess the relative engulfing and degrading capacities of the phagocytic cells. Smears and cell counts were also made. The results showed that these phagocytes were still very active immediately after exposure to doses as high as 50,000 r. Thus, for example, only a 10% inhibition in the engulfing index was detected immediately after 50,000 r. However, when the cells were cultured for 6, 12, or 24 hr after x irradiation before they were exposed to the target RBC, their phagocytic potential decreased progressively if the x-ray dose was greater than 800 r. This decrease can be correlated with death of phagocytic cells during the prephagocytic culture period following x irradiation but not with the decrease in the phagocytic capacity of the surviving phagocytes.

Discussion. – The concept that active phagocytes are involved in the initial step of antibody formation either by transferring the degraded antigens or their information RNA to receptor lymphoid cells, or by transforming to functional antibody-forming lymphoid cells after phagocytosis of the antigen, has become very popular. The results presented here demonstrate that the process of phagocytosis is extremely radioresistant (e^{-1} dose of $\sim$1500 r). In contrast, previous studies in this laboratory[1] have shown that the initiation of antibody formation is highly radiosensitive (e^{-1} dose of $\sim$70 r). It would appear, therefore, that if phagocytes are involved in the initiation of antibody formation, the radiosensitive process must be the postphagocytic "cellular transformation" or the "information transfer" process.

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Since the system is a relatively simple one, it could serve as a prototype for the study of sodium-potassium specificities in biology.

10.6 HELIX-COIL TRANSFORMATIONS OF POLYURIDYLIC ACID

R. A. Brown

The helix-coil transformations of polyuridylic acid have been studied in a thermostated Beckman DU spectrophotometer as a function of the solution environment. The melting temperature is dependent upon the types of cation and anion present. In 0.15 *M* solutions of sodium, potassium, and cesium chlorides, the melting temperatures of a polyuridylic acid solution were <1, 3.0, and 5.9°C respectively. In solutions of moderate ionic strength, the stability of the DNA double helix is not strongly affected by alcohols. At low ionic strength and high alcohol concentration, the melting temperature of DNA is lowered. In contrast to the results obtained with DNA, the melting temperature of the polyuridylic acid helix-coil transformation is increased by the addition of alcohol. In a 0.15 *M* CsCl solution, the addition of 20% ethanol raises the melting temperature from 5.9° to 11.3°C. These and other results suggest that charge-charge or dipole-dipole interactions rather than hydrophobic bonding are the most important factors in stabilizing the polyuridylic acid helix.

Molecular weight data obtained in the Beckman model E by means of short-column equilibrium as a function of KCl concentration suggest that the helix-coil transformations are quite complicated. Molecular weight changes may take place without an accompanying change in ultraviolet absorption. The data are best explained in terms of a mixture of two types of helices, one containing a single strand folded back on itself and the other containing two strands. An optimum condition for minimizing double-strand formation appears to be 0.30 *M* KCl.

The above data have several biological implications. Other investigators have observed that results obtained with amino-acid-incorporating systems are dependent upon the experimental conditions. These physical-chemical observations may have a bearing on their problem. The helix-coil transformation of polyuridylic acid has been shown to have a sodium-potassium specificity.

10.7 IMMUNOLOGICAL STUDIES WITH THE GRASSHOPPER NEUROBLAST

Mary E. Gaulden J. F. Albright
T. Makinodan

Two features of the immune response attract the attention of the cell biologist concerned with the mitotic process and with the function of cell structures. (1) Antigenically stimulated cells divide at a more rapid rate (in some cases at twice the rate) than do cells not exposed to antigen; in fact, antigen may cause nondividing cells to undergo considerable morphological differentiation and to divide.[1] (2) The first demonstrable appearance of antibody in a cell, as determined by the fluorescent-tagged antibody technique, occurs in the nucleolus.[2]

The production of a specific protein by the nucleolus is of obvious interest in view of the fact that this structure occurs at a specific site on a specific chromosome. An equally interesting question concerns the nature of the apparent relationship between stimulation of mitotic activity by a "foreign" protein and the production of antibody. Answers to these questions are of value not only from an immunological point of view but are even more exciting from the standpoint of gaining some insight into the molecular mechanisms of the processes of aging and of differentation and development.

Experimental attack on these questions would be greatly enhanced by use of a large cell that could be easily cultured and that has a well-defined mitotic cycle with cell structures visible in the living state. These conditions are admirably met by the neuroblast cell of the grasshopper embryo. In addition, the embryos of some species of grasshopper have, at a given point in development, a diapause period, a temporary but complete cessation of cell division, that might provide a good test system for the mitogenic action of specific antigens. The "breaking" of diapause, that is, the stimulation of dormant cells to undergo mitosis, is a vital aspect of development in these and other organisms.

One difficulty of some magnitude to be encountered in the use of the grasshopper for immunological studies is that an immune response as defined in mammals has not been unequivocally demonstrated in any animal below the vertebrates. There are, however, suggestive results of a positive nature in insects that encourage us to explore the problem.[3-5]

Some preliminary experiments designed to determine whether the neuroblast would show any reaction to antiserum have been performed. Antiserum was made by injecting into four rabbits a mixture of a 0.15% saline solution and groundup second instar nymphs of the grasshopper *Chortophaga viridifasciata* (De Geer). An interfacial test revealed a weak titer of antibody in serum from one animal (designated No. 1) and a good titer in the remaining three animals, the strongest antiserum giving an interface at a dilution of 1:256. The presence of antibody and the relative amounts of antibody in the sera were confirmed by the Ouchterlony gel diffusion test.

The first antiserum obtained (from animal No. 1) was examined by immunoelectrophoresis and run against extracts from both nymphs and embryos. Three or four weak arcs could be distinguished, the positions of one arc differing for embryo and nymph extracts. The other three antisera with higher titers of antibody will soon be examined immunoelectrophoretically to detect and identify the individual components.

The antiserum obtained from animal No. 1 was dialyzed against culture medium and used undiluted for preparing cultures of 14-day-old (26°C) *Chortophaga* embryos.[6] The embryo cultures were studied with bright-field illumination and the oil immersion lens of a compound microscope enclosed in a box maintained at 38 ± 1.5°C. The neuroblasts in the embryos were examined within a few minutes after culture preparation and were followed for several days.

After 2 hr of continuous culture exposure to antiserum (AS), four distinct cytological aberrations were observed in the neuroblast.

1. Breakdown of the nuclear membrane at very late prophase was completely inhibited; the chromosomes, randomly distributed in the nucleus, continued contracting and assumed the appearance of much shortened metaphase chromosomes. The two nucleoli, which in untreated cells disappear at the beginning of this stage, persisted and remained attached to their chromosomes. Several hours later, each chromosome became a spherical, refractile body, and the cell disintegrated at about 12 hr.

2. The spindle in metaphase cells was much reduced in size. At 3 hr the chromosomes were highly refractile and clumped in the center of the cell; no cytoplasm could be distinguished. At varying times, cells in this condition disintegrated.

3. Chromosomes of cells in early and middle telophase did not undergo the "dispersion" typical for these stages, but remained condensed and became highly refractile. Two chromatids could be readily distinguished in each chromosome. Nucleolar formation was inhibited in most cells. No growth of nucleus or cell was observed.

4. The mature nucleoli of cells in stages from late telophase to midprophase spherated and broke into several parts that eventually became randomly distributed in the nucleus.

By 12 hr of exposure, all mitotic progress had ceased and many cells had disintegrated. By 24 hr half, and by 48 hr all, the cells in an embryo were empty or had disappeared. The cells most sensitive to the cytotoxic action of the AS were those in the small appendage on the first abdominal segment.

Thus it appears that AS causes cessation of mitosis in the neuroblast by interfering initially with nucleolar and spindle structure and function, with nuclear membrane breakdown, and with normal telophase development of chromosomes. The cytotoxic effects observed with AS contrast strikingly with the almost total absence of such effects following exposure of the grasshopper embryo to large doses of a variety of physical and chemical agents.

To determine whether some or all of the effects observed with AS were caused solely by the proteins in normal rabbit serum (NRS), embryos were observed in NRS dialyzed against culture medium. All effects were observed, but their appearance was much delayed and their magnitude much reduced. After three days of exposure, only a few cells were dead; not until seven days were all of them dead.

Dilution experiments with both AS and NRS revealed that a dilution of AS 1:999 did not eliminate its effects. This observation assumes even more significance in view of the fact that the AS sample used had the lowest titer of antibody from four rabbits. On the other hand, dilution of NRS 1:9 almost completely eliminated its effects.

As soon as grasshopper embryos are again available in the spring, experiments will be done with heated AS and NRS to determine how much of the effects are due to complement.

To determine whether any foreign protein would cause cytological effects similar to those observed with AS, embryos were exposed to a 5% solution of bovine serum albumin in culture medium. Not only was there complete absence of cytological or mitotic abnormalities, but such cultures grew better than the control ones in culture medium alone.

Prior to performing experiments with fluorescent-tagged antibodies to determine intracellular sites of antigen-antibody complexes, we have made frozen sections of unfixed embryos and examined them with ultraviolet light. The cells show no autofluorescence.

A search of the literature has, to date, revealed no observations similar to those described above, excepting the cytotoxic action of AS. The non-immunologist is impressed with the lack of attention that has been given to basic cytological aspects of the immune response.

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11. Pathology and Physiology

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Pathological Effects of Neutrons, High-Energy Radiation, and Chemicals (11.1–11.3)

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11.1 LIFE SHORTENING IN RF FEMALE MICE IRRADIATED WITH 14-Mev NEUTRONS

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Introduction. – Preliminary results have been reported previously on RF female mice irradiated acutely with sublethal doses of 14-Mev neutrons (0, 50, 100, 200, and 400 rads).[2] The results presented here cover life shortening in the 30-day survivors (98.2% of those exposed). Other long term effects (body weight, hematology, neoplasia, lens opacities, bone changes, etc.) are to be reported later.

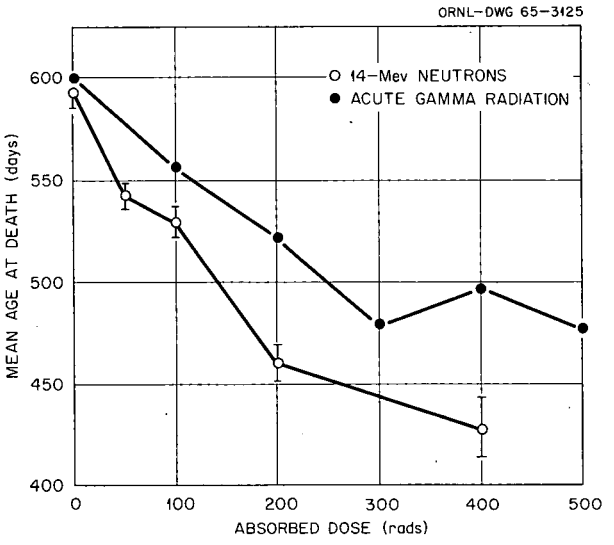

Fig. 11.1.1. Mean Age of Death from All Causes.

Results and Discussion. – The mean age of death from all causes decreased with increasing dose, to a somewhat greater extent than with acute gamma radiation (Fig. 11.1.1). The mean age at death in all treatment groups for animals with neoplasms was older, and for animals without neoplasms younger, than the mean age at death from all causes (Table 11.1.1).

From these results, it may be inferred that 14-Mev neutrons are at least twice as effective as x rays in reducing the longevity of the mouse and that their life-shortening effects cannot be explained solely on the basis of carcinogenesis. These results also imply the importance of high-LET collision secondaries in the biological action of 14-Mev neutrons.[3]

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11.2 DURATION OF DONOR SENSITIZATION IN THE PARENT-TO-F_1 FOREIGN SPLEEN REACTION

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Introduction. – The morbidity and mortality caused by injection of parental spleen or marrow cells into irradiated F_1 recipient mice may be

Table 11.1.1. Effects of 14-Mev Neutrons on Life Span

Absorbed Dose (rads)	Number of Animals	Mean Age at Death (days + S.E.)		
		All Causes	With Neoplasms	Without Neoplasms
0	519	586 ± 7.0	610 ± 8.4	544 ± 13.4
50	530	542 ± 7.0	555 ± 7.7	505 ± 17.6
100	332	528 ± 8.8	537 ± 9.4	509 ± 31.4
200	227	459 ± 10.6	473 ± 11.3	398 ± 38.9
400	116	428 ± 14.8	449 ± 16.8	366 ± 40.0

Table 11.2.1. Mortality of $(101 \times C_3H)F_1$ Mice Injected with C_3H Spleen Cells After 500 r Whole-Body X Irradiation

Spleen Donor		Recipients			
Strain	Time of Sensitization Prior to Use	Number	Mortality (%) At 30 Days	Mortality (%) At 90 Days	Mean Time of Death (days after treatment)
None		66	6	15	22
$(101 \times C_3H)F_1$		77	12	23	40
C_3H		46[a]	4	22	50
C_3H	28 days	57	70	100	23
C_3H	12–17 months	50	38	80	32

[a]Includes 20 animals given spleen cells from unsensitized donors held for 12–17 months before use. The mortality in this group did not differ from that seen when the donors were young adults.

enhanced by presensitization of the donor to the reciprocal parent, even in those combinations of parents identical in *H-2* antigens.[1,2] To determine how long the effects of such presensitization of the donor last, we have tested some donors as late as 17 months after sensitization and are presenting our results herewith.

Results and Discussion. – Spleen cells from sensitized donors, administered in doses of approximately 15×10^6 cells/recipient, were effective in producing a strong foreign spleen reaction as long as 17 months after sensitization (Table 11.2.1). This indicates a long duration of the state of hypersensitivity in the donor spleen cells responsible for the graft-vs-host reaction. The effect does not necessarily indicate, however, the survival of individual cells (e.g., "long-lived" lymphocytes) for this entire period.

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11.3 VIRAL INDUCTION OF MYELOID LEUKEMIA: INHIBITION BY ESTROGEN

V. K. Jenkins T. T. Odell, Jr.

Introduction. – Viral and sex factors have been demonstrated in the pathogenesis of radiation-induced myeloid leukemia in RF mice. This report concerns the effects of sex hormones on viral transmission of the disease.

Results and Discussion. – Estrogen, testosterone, and hormone vehicle (sesame oil) were injected into gonadectomized and intact RF mice of both sexes, 96 mice per group. Gonadectomy was performed at three weeks of age, and hormone treatment was begun at four weeks. Estradiol benzoate was injected intramuscularly, weekly, 33 μg in 0.2 cc of sesame oil. Testosterone propionate was injected intramuscularly, weekly, 1.0 mg in 0.2 cc of sesame oil. Vehicle (sesame oil) was injected intramuscularly, weekly, in the same volume. Cell-free ultracentrifugates were prepared from mice with serially passaged myeloid leukemia as described earlier[1] and were injected into equal numbers of four-week-old recipients in each treatment group.

Myeloid leukemia developed within 90 days in 16–20% of intact and gonadectomized males given testosterone or vehicle (Fig. 11.3.1) and in 12–14%

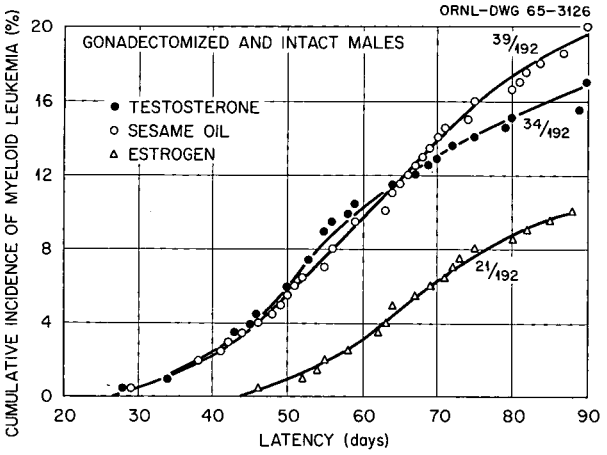

Fig. 11.3.1. Cumulative Incidence of Myeloid Leukemia in Males as Influenced by Type of Hormone Injected. Results for intact and gonadectomized animals are pooled.

of comparably treated females. In animals given estrogen, the incidence was 9 and 12% in intact and gonadectomized males (Fig. 11.3.1), respectively, and 11 and 5% in intact and gonadectomized females respectively.

In summary, the induction of leukemia was not affected by either testosterone or gonadectomy, but was inhibited by estrogen. The mechanism of the inhibition is under study.

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11.4 MATURATION OF RAT MEGAKARYOCYTES STUDIED BY MICROSPECTROPHOTOMETRIC MEASUREMENTS OF DNA

T. T. Odell, Jr. C. W. Jackson

Introduction. – The maturation process of cells of the megakaryocyte series is not well understood. It is generally accepted that nuclear division occurs without cytoplasmic division during megakaryocyte maturation, with the result that nuclei of mature megakaryocytes contain several times the normal diploid chromosome complement of the species. There has not been any precise method of grouping megakaryocytes into maturation stages based on their appearance under the light microscope, and it, therefore, seemed that measurement of the quantity of DNA in individual megakaryocytes might provide a systematic means of classification. To this end, we have measured, microspectrophotometrically, the DNA content of megakaryocytes in Feulgen-stained preparations of smears of rat bone marrow.

Results and Discussion. – Smears of bone marrow of eight male Sprague-Dawley rats were treated with the Feulgen reaction to stain nuclear DNA. Blood smears of some of the rats were similarly treated. The DNA of individual cells was measured by the two-wavelength microspectrophotometric method. Plotting frequency against increasing quantity of DNA of megakaryocytes produced a multimodal curve with peak modal values fitting the expression $2^X N$, where N equals an estimate of the haploid chromosome number. The ploidy of the various groups of megakaryocytes was determined by comparing the megakaryocyte values with values obtained by measuring the DNA of lymphocytes, monocytes, and other marrow cells which were assumed to be diploid cells. Frequencies of $4N$, $8N$, $16N$, and $32N$ megakaryocytes were, respectively, 1.5, 10.5, 71, and 17%. These results indicate that division of the nuclei of multinucleated (polyploid) megakaryocytes is synchronous. In addition, if it is assumed that all megakaryocytes follow the same maturation process and pass through all stages, then the relative number of cells of each ploidy indicates the relative length of time spent in each corresponding stage. On this basis, the largest percentage of the maturation time is spent in the $16N$ stage. Other interpretations, however, are also possible.

Measurement of DNA by microspectrophotometry provides a means of classifying megakaryocytes into groups. We expect this method to be of considerable value in future studies of the life cycle of megakaryocytes and studies of the mechanisms that regulate megakaryocytes and platelets.

11.5 CONCERNING THE "STRENGTH" OF THE *H-2* CONTROLLED HYBRID RESISTANCE

G. Cudkowicz L. C. Satterfield

Introduction. – F_1 hybrid mice that are heterozygous at the D region of the *H-2* locus resist the

growth of transplanted marrow cells when the donor is homozygous for the $H\text{-}2^b$ allele.[1–3] We have estimated the strength of this resistance by determining (1) the number of donor cells necessary to override it and thereby to repopulate the spleen of the recipient, and (2) the extent to which the resistance is weakened by exposure of the recipient to x rays.

Results. – Graded doses of parental marrow cells were grafted into isogenic and into F_1 recipients exposed to 700–900 r of x rays. Five days later, the extent of repopulation of the recipient spleen was estimated by the ^{131}IUdR uptake method. Data plotted in Fig. 11.5.1 indicate that, in general, the number of cells required to repopulate F_1 recipients was higher than the number required for repopulation of isogenic recipients. Differences in strength of hybrid resistance were noticeable, however, on comparison of the cell-dose responses in hybrids of different genetic constitution. From the data it seems that resistance is strongest in hybrids whose parents share C57BL genetic background and weakest in hybrids whose parents share C3H and A genetic backgrounds, irrespective of the *H-2* alleles of the heterozygotes. Furthermore, the strength of the hybrid resistance in several strain combinations appears to be of the same order of magnitude as the resistance of mice to allogeneic marrow grafts. For example, C3H mice are weakly resistant to B10 marrow grafts,[4] whereas DBA/2 mice are strongly resistant, as shown in Fig. 11.5.2.

(C3H × C57BL)F_1 mice and DBA/2 mice were exposed to x rays and grafted thereafter with 10^6 or with 5×10^6 nucleated C57BL marrow cells. Both types of recipients resisted the grafting of 10^6 cells after exposures ranging from 600 to 1000 r of x rays (Fig. 11.5.2). A progressive loss of resistance was noticeable, however, with increasing exposure in the DBA/2 and in the hybrid mice when 5×10^6 marrow cells were grafted (Fig. 11.5.2).

Conclusion. – The strength of hybrid resistance to parental marrow grafts, as measured by the number of grafted cells required to override the

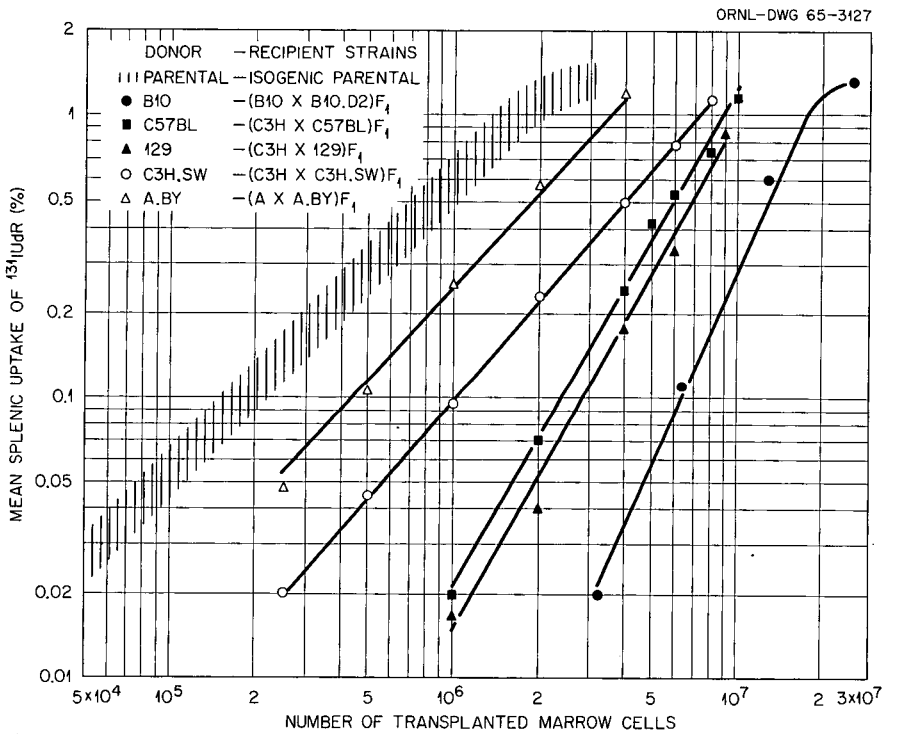

Fig. 11.5.1. Recipient Spleen Repopulation by $H\text{-}2^b$ Homozygous Marrow Cells Transplanted into $H\text{-}2^b$ Heterozygous F_1 Mice Exposed to 700–900 r of X Rays. Displacement to the right of dose-response curves measures the strength of resistance.

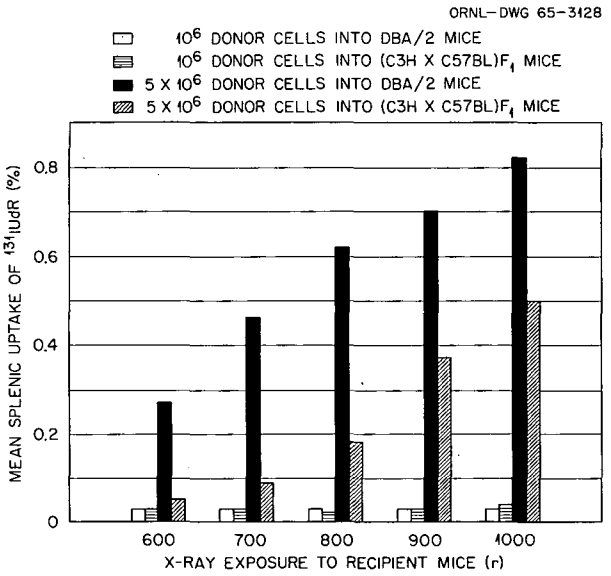

Fig. 11.5.2. Radiation Sensitivity of the Resistance to C57BL Marrow Grafts.

resistance, or by the dose of x rays required to weaken the resistance, appears to be of the same order of magnitude as the resistance to grafted marrow found in some *H-2* incompatible donor-host combinations. It appears, furthermore, that the strength of the *H-2* controlled hybrid resistance is affected by the hybrid's genetic background.

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11.6 A SECOND CASE OF HYBRID RESISTANCE TO PARENTAL MARROW GRAFTS: ASSOCIATION WITH SEX

G. Cudkowicz L. C. Satterfield

Introduction. – In the course of previous studies on the *H-2* controlled hybrid resistance to parental marrow grafts, female F_1 hybrids between the congenic lines of mice C57BL/10ScSn or B10 ($H\text{-}2^b$) and B10.D2 ($H\text{-}2^d$) were found to resist the transplantation of B10 parental marrow cells but not of B10.D2 marrow cells.[1] We noticed, however, that B10.D2 marrow grew better on transplantation into female F_1 hybrids than into male hybrid recipients. The finding was unusual, for the two parental strains entering the F_1 cross were congenic with respect to histocompatibility, although it was known that strain B10.D2 differed from strain B10 by an allele at the *H-2* locus, as well as at the locus for hemolytic complement *Hc*, and that both alleles foreign to the B10 strain were derived from strain DBA/2 ($H\text{-}2^d$).[2] This investigation was undertaken to establish to what extent male F_1 hybrids were resistant to parental B10.D2 marrow grafts and whether such resistance was due to histocompatibility determined by genes on the X chromosome[3] or by interaction between alleles on the X and Y chromosomes derived, respectively, from strains B10 and DBA/2.

Results. – DBA/2 or B10.D2 marrow (5×10^5 cells) was transplanted into x-irradiated (700 r) isogenic recipients, B10.D2 × B10 and reciprocal F_1 hybrids, DBA/2 × B10.D2, and C57BL/6 × DBA/2 F_1 hybrids. The growth of the grafted marrow was determined five days later by measuring the uptake of ^{131}IUdR in recipient spleens. Recipient mice were designated "resistant" if the splenic uptake of ^{131}IUdR by the grafted marrow was one-third or less of the uptake by an identical marrow graft in "susceptible" isogenic recipients. Although marrow from DBA/2 and B10.D2 donors of either sex grew well in isogenic females and males, in all types of F_1 hybrid females, and in DBA/2 × B10.D2 males, it failed to grow well in C57BL/6 × DBA/2, B10 × B10.D2, and reciprocal F_1 males (Table 11.6.1). This latter finding indicated that the resistance of hybrid males was not ascribable to their lack of the DBA/2 or B10.D2 X chromosome.

Conclusion. – The similar behavior of DBA/2 and B10.D2 marrow cells on transplantation into irradiated F_1 hybrid recipients and, furthermore, the lack of resistance of hybrids between these two strains of mice suggest that the inability of B10.D2 marrow to grow well on transplantation into certain male hybrids is acquired together with other traits of DBA/2 origin at the loci *H-2* and *Hc*. This sex-associated hybrid resistance to parental marrow grafts resembles, in many ways, the resistance of

Table 11.6.1. Resistance and Susceptibility to Parental Marrow Grafts in Female and Male F_1 Hybrids

Strain of Marrow Donor	Strain and Sex of Recipients	Classification
DBA/2, ♀ and ♂	DBA/2 × B10.D2 ♀	Susceptible
	DBA/2 × B10.D2 ♂	Susceptible
	C57BL/6 × DBA/2 ♀	Susceptible
	C57BL/6 × DBA/2 ♂	Resistant
	B10 × B10.D2 ♀	Susceptible
	B10 × B10.D2 ♂	Resistant
	B10.D2 × B10 ♀	Susceptible
	B10.D2 × B10 ♂	Resistant
B10.D2, ♀ and ♂	DBA/2 × B10.D2 ♀	Susceptible
	DBA/2 × B10.D2 ♂	Susceptible
	B10 × B10.D2 ♀	Susceptible
	B10 × B10.D2 ♂	Resistant
	B10.D2 × B10 ♀	Susceptible
	B10.D2 × B10 ♂	Resistant

H-2 heterozygotes to marrow grafts from *H-2*^{b} homozygous donors,[1] conceivably representing another case of genetic interaction of mouse histocompatibility factors, possibly between genetic determinants on X and Y chromosomes.

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11.7 STEM CELLS AND "LYMPHOCYTES" IN MOUSE BONE MARROW

M. Bennett G. M. Shearer
G. Cudkowicz

Introduction. – The proliferative activity of mouse bone marrow has been correlated with its content of small and medium-sized "lymphocytes."[1–3] To determine whether or not this correlation persists under a variety of conditions, the cell composition of mouse bone marrow was altered experimentally in vivo by (1) hypertransfusion with isogenic erythrocytes, (2) serial transplantation through three isogenic recipients, (3) antigenic stimulation with allogeneic leukocytes, and (4) administration of cortisol. The marrow was also altered in vitro by filtration through glass-wool columns to remove cells other than "lymphocytes."

Results. – Adult (C3H × C57BL)F_1/Cum mice were used throughout. After each of the in vivo treatments, the mice were killed and marrow cells were harvested from one femur to determine marrow cellularity. The assay for proliferative competence consisted of infusing 2–5 × 10^5 cells into isogenic test-recipient mice exposed to 800–900 r of x radiation and of estimating, five days later, the induced splenic hyperplasia by means of the ^{131}IUdR uptake method. The donor marrow cells were examined microscopically on air-dried smears. Polycythemia failed to change either "lymphocyte" concentration or proliferative activity of marrow; serial transplantation reduced both; antigenic stimulation with allogeneic leukocytes and cortisol administration reduced "lymphocyte" concentration but not proliferative competence (Table 11.7.1).

Marrow cells were harvested from polycythemic, cortisol-treated, and from normal age-control mice for filtration over glass-wool columns as previously described.[2] Filtered marrow cell suspensions were more potent than unfiltered marrow in repopulating the spleens of recipients. The frequency of "lymphocytes" in the filtered marrow increased and seemed to account for the increased proliferative activity, irrespective of the treatment received by the donor mice (Table 11.7.2).

Conclusion. – Marrow "lymphocytes" appear to be a functionally heterogeneous population of cells. However, a small proportion of them are correlated with hemopoietic cell production, presumably by acting as undifferentiated precursor cells.

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Table 11.7.1. Concentration of Small and Medium-Sized "Lymphocytes," Cellularity, and Proliferative Capacity of Mouse Bone Marrow Altered by Polycythemia, Leukocyte Transfusion, Serial Transplantation, and Cortisol Administration

Treatment	Age Controls	Polycythemia	Isogenic Leukocytes	Allogeneic Leukocytes	Serial Transplantation	Cortisol
Mean percentage of small and medium-sized "lymphocytes"	29.9	33.6	30.0	9.9	7.8	8.0
Mean number of nucleated cells in one femur ($\times 10^6$)	22.4	23.0	23.1	22.2	3.5[a]	11.1
Mean uptake of ^{131}IUdR in recipient spleens promoted by 10^5 donor cells[b]	0.25	0.28	0.26	0.28	0.10	0.35

[a]Because of poor marrow cellularity, cells were pooled from two femurs, two tibiae, and two humeri.
[b]Uptake values for spleens of mice injected with marrow are given as the percentage of the total ^{131}IUdR radioactivity administered, above the percentage retained in spleens of irradiated control animals not injected with marrow.

Table 11.7.2. Frequency of Small and Medium-Sized "Lymphocytes" and Proliferative Capacity of Mouse Bone Marrow Cells

	Marrow of					
	Age-Control Donor Mice		Polycythemic Donor Mice		Cortisol-Treated Donor Mice	
	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered
Percentage of small and medium-sized "lymphocytes"	27.1	42.2	26.5	45.2	4.5	14.0
Mean uptake of ^{131}IUdR in recipient spleens promoted by 10^5 injected cells:[a]						
All nucleated cells	0.27	0.36	0.23	0.39	0.26	0.59
Small and medium-sized "lymphocytes"	0.84	0.85	0.86	0.86	3.09	2.93

[a]Uptake values for spleens of mice injected with marrow are given as the percentage of the total ^{131}IUdR radioactivity administered, above the percentage retained in spleens of irradiated control animals not injected with marrow.

11.8 INFLUENCE OF AGE AND THE GERM-FREE STATE ON MOUSE PERIPHERAL BLOOD CELLS

H. E. Walburg, Jr. E. I. Mynatt
D. G. Gosslee[1]

Introduction. – Examination of germ-free animals has revealed them to have a significantly smaller amount of lymphatic tissue and smaller number of peripheral blood leukocytes than their counterparts.[2] With the discovery that the generation cycle of the intestinal epithelium of germ-free mice is significantly longer than that found in conventional mice,[3] it has become important to define the manner by which the microbial flora influences cellular renewal. Since the peripheral blood may

indicate hematopoietic activity, preliminary experiments were performed to evaluate the effect of the germ-free state on the peripheral blood cells of mice of different ages.

Results and Discussion. – No significant differences were observed between germ-free and conventional mice younger than 16 days. The total erythrocyte and leukocyte counts, as well as the lymphocyte and neutrophil counts, remained relatively stable during this period. As the age of the mice increased beyond this point, there was a gradual rise in the number of erythrocytes and leukocytes in both germ-free and conventional mice (Fig. 11.8.1). The rate of increase of erythrocytes did not differ in the germ-free and conventional mice. There was, however, a significant difference ($P < 0.01$) in the slopes of the curves obtained for total leukocytes, lymphocytes, and neutrophils between germ-free and conventional mice (Figs. 11.8.1 and 11.8.2). In all of these groups, the age-related rise in number of peripheral cells was more rapid in the conventional mice than in the germ-free mice. The amount of variability also increased with age in both germ-free and conventional mice, but was greater in the conventional mice. The monocytes constituted only a small proportion of the total leukocytes found in the peripheral blood and did not seem to be affected by the microbial flora. The eosinophils, also few in number, were frequently seen in the conventional mice but almost never in the germ-free mice.

Most of the effect of microbial flora on the number of circulating leukocytes was manifest through changes in the number of lymphocytes, since they constituted about two-thirds of the total leukocytes in the peripheral blood. The reduced number of lymphocytes in the germ-free mice was probably correlated with a reduced number of antibody-containing cells, since this type of cell has been shown to be deficient in the lymph nodes of germ-free mice.[4] It is noteworthy, however, that even in the germ-free mice there was an age-related increase in the number of lymphocytes.

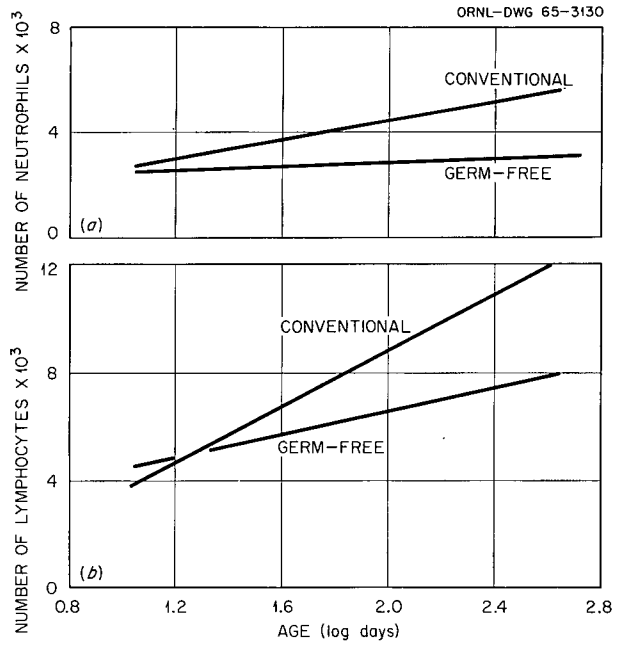

Fig. 11.8.2. (*a*) **Linear Regression of Peripheral Neutrophils in Germ-Free and Conventional ICR Mice in Relation to the Logarithm of Age.** (*b*) **Linear Regression of Peripheral Lymphocytes in Germ-Free and Conventional ICR Mice in Relation to the Logarithm of Age.**

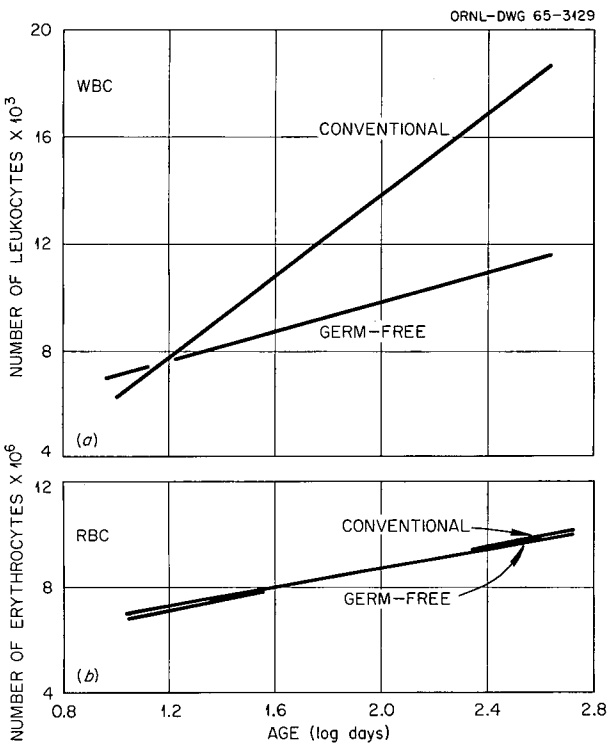

Fig. 11.8.1. (*a*) **Linear Regression of Peripheral Leukocytes in Germ-Free and Conventional ICR Mice in Relation to the Logarithm of Age.** (*b*) **Linear Regression of Peripheral Erythrocytes in Germ-Free and Conventional ICR Mice in Relation to Logarithm of Age.**

Whether this resulted from a constant but relatively low level of antigenic stimulation from antigens in the food and bedding, or from a physiological aging phenomenon, is under study. The smaller number of neutrophils in the germ-free mice may reflect an absence of infection. The cellular turnover of blood cells in relation to the incidence of various types of leukemia is under study.

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11.9 IN VITRO CYTOPATHOGENIC EFFECTS ASSOCIATED WITH THE RAUSCHER LEUKEMIA VIRUS

R. L. Tyndall — Ernestine Teeter
J. G. Vidrine — H. E. Walburg, Jr.
R. F. Zeigel[1] — M. A. Fink[1]

Introduction. – Cytopathic effects (CPE) observed in mouse cell cultures infected with Rauscher leukemia virus included cytoplasmic vacuolization, eosinophilic inclusion bodies, and marked variation in the size, shape, and intensity of staining of cell nuclei.[2] Uninfected control cultures showed no CPE. Since leukemia viruses propagated in tissue cultures have heretofore failed to elicit CPE, studies were undertaken to determine if the observed CPE in our cultures was associated with the Rauscher leukemia virus per se.

Results and Discussion. – To determine if the CPE might be related to inadvertent infection with mouse viruses other than the Rauscher virus, infected cultures were injected intraperitoneally into adult germ-free RF/Unf mice. These mice are resistant to the Rauscher leukemia virus, but sensitive to infection with other mouse viruses. Three months after injection, the mice were bled and their serum tested for antibodies against the following mouse viruses: polyoma, K, reo. 3, Sendai, mouse poliovirus, mouse hepatitis, and mouse adenovirus. All such tests were negative. These results indicated the cultures infected with the Rauscher leukemia virus and showing CPE were free of the aforementioned nonleukemic mouse viruses. Electron microscopic examination of these cultures revealed large numbers of leukemia virus particles. Many of these particles were associated with, and budding from, the cell membrane, as expected; however, atypically large numbers of intracytoplasmic particles were also seen. Whether this anomaly is a cause or effect of the CPE has yet to be determined. Examination of infected and control cultures was carried out with fluorescein-labeled rabbit anti-Rauscher virus antisera, according to the methods described by Fink and Malmgren.[3] Results to date show fluorescence of both cytoplasmic and nuclear inclusions in cells of the infected cultures. Such fluorescence is indicative of Rauscher virus antigen. No fluorescence has been detected in uninfected control cells.

Failure to detect nonleukemic mouse viruses in the infected cultures, together with evidence of Rauscher virus and Rauscher virus antigen in the nuclei and cytoplasm of cells showing CPE, strongly suggests an association between the observed CPE and the Rauscher virus per se. Evidence of this association strengthens the possibility of developing an in vitro assay for the murine leukemias.

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11.10 OBSERVATIONS OF THE RNA METABOLISM OF LEUKEMIC CELLS

R. L. Tyndall — Ernestine Teeter
V. K. Jenkins

Introduction. – The RNA metabolism of cells infected with poliovirus and other cytocidal viruses has been studied in detail.[1] Several hours after infection the synthesis of RNA in such cells is resistant to the inhibitory effects of both actinomycin and puromycin, and the RNA synthesized shows high levels of the AU dinucleotide relative

Table 11.10.1. Characteristics of RNA-^{32}P Metabolism in Normal and Leukemic Cell Cultures

	Specific Activity[a]			Oligonucleotide Ratios[b]		
	Untreated	Actinomycin Treated	Puromycin	U/AU	U/GAC	U/GC
Myeloid leukemia spleen cultures	34,000	4700	1210	2.35	1.24	1.28
Reticulum cell sarcoma spleen cultures	6,600	580	2200	1.91	0.77	1.06
Normal spleen cultures	3,800	635	1210	1.71	1.68	1.33

[a]CPM RNA-^{32}P/OD_{260} total RNA. Average of three experiments.
[b]Determined by densitometric analysis of fingerprint patterns (see ref. 2). Average of three experiments.

to its levels of GC and GAC oligonucleotides.[1–3] These findings suggest that the RNA is of messenger type and polymerized by an RNA-dependent RNA polymerase.

There is little comparable information concerning the changes in RNA metabolism of cells infected with leukemia viruses. The present study was undertaken to determine some gross characteristics of RNA metabolism in reticulum cell sarcomas and myeloid leukemias resulting from the Rauscher and myeloid leukemia viruses respectively.

Results and Discussion. – Leukemic spleens from RF mice infected with either the Rauscher or myeloid leukemia viruses were minced aseptically, and the resultant suspensions were cultured in Eagle's medium containing 20% horse serum and a fourfold concentration of amino acids and vitamins. Comparable cultures from normal RF mouse spleens were also prepared. Each culture was divided into three groups. Actinomycin (1 μg/ml) was added to one group, and puromycin (50 μg/ml) to a second group, 1 hr after explantation. The third group was untreated. Phosphate labeled with ^{32}P (50 μc/ml) was added to all groups 2 hr after explantation. Seven hours after explantation RNA was extracted from all cultures with phenol at 60°C.[4] Specific activities and fingerprint patterns of the resultant RNA's were determined.[2] The RNA from both leukemic cell cultures (Table 11.10.1) showed a higher turnover rate than did normal spleen cell RNA. The RNA synthesis in myeloid leukemia cells showed an extremely high specific activity as well as an extreme sensitivity to puromycin. Actinomycin D inhibited 80–90% of the RNA synthesis in both leukemic cell cultures. The RNA synthesized in both leukemic cell cultures was characterized by high levels of GC and GAC oligonucleotides relative to the levels of AU dinucleotide.

Although differences in the sensitivity of RNA synthesis to inhibition by puromycin were evident between the two kinds of leukemic cells, both cultures synthesized predominantly DNA-dependent RNA of the ribosomal type. They thus contrasted sharply with cells infected by cytocidal viruses in the type of RNA they synthesized. The patterns of RNA synthesis in cells transformed by tumor viruses and other types of carcinogens remain to be determined.

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11.11 DISCONTINUOUS, VERTICAL FLAT-BED ELECTROPHORESIS IN ACRYLAMIDE GEL

R. C. Allen D. J. Moore

Introduction. – We have previously reported on the separation and quantitation of esterase isozymes in acrylamide gel with the disk electrophoretic technique.[1,2] However, it is not possible to do comparative staining on a single sample with the disk technique because the shape of the gel and its physical properties preclude accurate vertical sectioning of the gel. On the other hand, gels prepared with the flat-bed technique of Raymond may be sectioned easily, although the resolution with this procedure is not as discrete as with the disk method. The following system embodies the advantages of both earlier techniques and, in addition, produces better resolution than does the disk technique, particularly in the region of the higher-molecular-weight proteins. Since available instrumentation for the densitometric analysis of stained reactions produced with this system was not suitable, a description of a prototype microscope densitometer is also given.

Results and Discussion. – Gels prepared from recrystallized monomer were cast in Tiselius cells in a manner similar to that used in the disk electrophoresis technique, with the exception that the whole system was cast in the reverse order; that is, a spacer gel cap was followed by the sample in 50% buffered sucrose, spacer gel, and then lower gel of a selected pore size. The last two discontinuities may be repeated, using different pore-size lower gels, thus producing a gradient system whereby larger-molecular-weight proteins may be trapped at any desired level in the gel. Electrophoretic separations were carried out in the lower gel for 1 hr at 10 ma. Following development of the esterase isozymes,[1,2] gels were equilibrated in glycerine, mounted on microscope slides in glycerine, covered with cover slips, and scanned densitometrically with a Leitz microscope spectrophotometer. The latter was modified by the addition of a substage drive mechanism and an integrating recorder in such a manner that isozyme bands separated by less than 1 mm could be both resolved and accurately quantitated.

Preliminary data on esterase isozymes of mouse plasma and organ extracts analyzed with this technique indicate that the plasma, liver, kidney, and probably adrenal have a similar total of esterase isozymes, numbering more than 20 and differing only in their quantitative organ distribution. This technique is of promise in further producing multiple discontinuities in the gel which will trap selected isozyme bands at various levels, depending on the gradient of pore sizes.

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12. Cell Physiology

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12.1 OSMOTIC PHENOMENA IN BACTERIOPHAGES T4B AND T4BO$_1$

S. P. Leibo Peter Mazur

The osmotic relations in bacteriophages T4B and T4BO$_1$ have been studied (1) as a factor in their response to freezing,[1] (2) in order to determine the effects of changes in the ionic environment, and (3) as a prerequisite to an investigation of the nature and role of internal water in the phage head.

Phages T4B and an osmotic-shock-resistant mutant, T4BO$_1$,[2] were diluted rapidly or slowly to various final salt concentrations subsequent to suspension in NaCl solutions from 0.5 to 3.0 *m*. They were also rapidly diluted after suspension in 6.0 *m* glycerol. Survival was assayed by standard methods. The results are shown in Table 12.1.1 and Figs. 12.1.1 and 12.1.2.

The survival of T4B following rapid dilution of a suspension in concentrated solutions is primarily dependent on an osmotic pressure differential between the internal environment of the phage head and the suspending medium. This explanation was first proposed by Anderson *et al.*[3] Two facts that support this hypothesis are shown in Table 12.1.1. (1) When $\Delta P/\Delta t \leqq 15$ atm/min, survival is 100% even when $\Delta P_{total} = 150$ atm. However, when $\Delta P/\Delta t = 150$ atm/min, survival is approximately 1%. (2) When a suspension of T4B in a concentrated solution is diluted rapidly, survival is low whether the solute is ionic (e.g. NaCl) or nonionic (e.g. glycerol).

There is, however, an additional salt concentration effect that has not previously been recognized. When T4B is suspended in salt solutions above 1.5 *m* and then diluted, survival first decreases precipitously due to the osmotic effect, but then continues to decrease gradually with decreasing final salt concentration (Fig. 12.1.2*a*). We suggest that this gradual decrease in survival is a reflection of the lack of neutralization of the phosphate groups of the phage DNA at very low ionic strengths. It may be analogous to the spontaneous melting of DNA at 25°C in very dilute salt solutions.[4] This suggestion is supported by

Table 12.1.1. Osmotic Shock of Phages T4B and T4BO$_1$

	Percent Survival	
	T4B	T4BO$_1$
3.0 *m* NaCl, rapid dilution	1.1	68.5
3.0 *m* NaCl, slow dilution	111.5	73.1
6.0 *m* glycerol, rapid dilution	0.9	99.3

the results with phage $T4BO_1$, in which the osmotic effect is not operative. That is, suspending $T4BO_1$ in NaCl solutions greater than 0.5 *m* and subsequently diluting it causes a marked decrease in survival with decreasing final salt concentration (Fig. 12.1.2*b*). We propose the following hypothesis to explain these results: When either of these phages is suspended in concentrated NaCl, NaCl enters the head and replaces

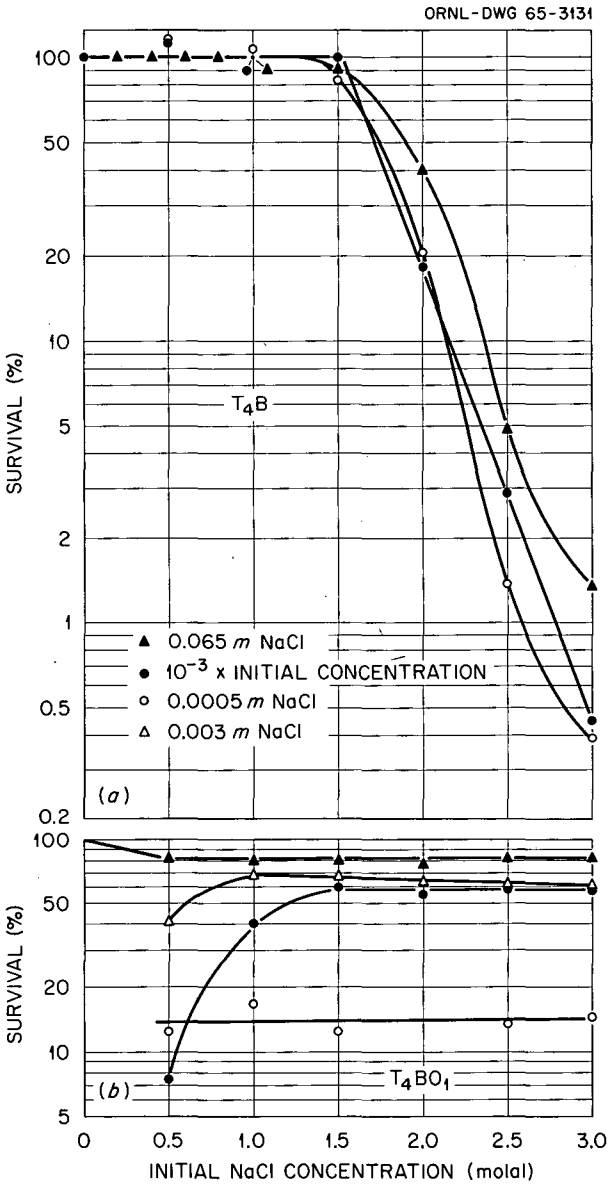

Fig. 12.1.1. Survival of Phages T4B and $T4BO_1$ as a Function of the Initial Molal Concentration of NaCl Prior to Dilution to Each of the Indicated Final Concentrations or to 10^{-3} × Initial Concentration.

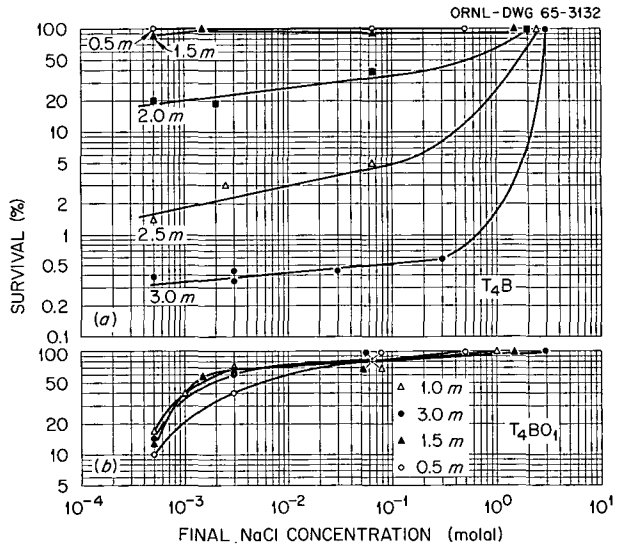

Fig. 12.1.2. Survival of Phages T4B and $T4BO_1$ as a Function of the Final Molal Concentration of NaCl to Which the Suspension Was Diluted Following Suspension in Each of the Indicated Initial Concentrations of NaCl.

polyamines and Mg^{2+}, which normally serve to neutralize the DNA charge. As a consequence of the well-documented differences in the permeabilities of the wild-type phage and its mutant,[5] the polyamines remain within the head of T4B but diffuse out of $T4BO_1$. When T4B is rapidly transferred into distilled water, water diffuses into the head more rapidly than the excess NaCl can diffuse out. The resulting osmotic pressure against the phage membrane produces osmotic shock. Osmotic shock does not occur in $T4BO_1$ because the NaCl can leave as rapidly as the water enters. Moreover, when the external Na^+ concentration is reduced to very low values, the Na^+ which remains in the head of both T4B and $T4BO_1$ is no longer able, because of its low binding affinity for DNA,[6,7] to neutralize the charges on the DNA effectively. The resulting repulsion of the phosphate groups produces an alteration in the structure of the usually highly ordered DNA and, therefore, a noninfective phage particle.

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12.2 FREEZING OF BACTERIOPHAGES T4B AND T4BO$_1$

S. P. Leibo Peter Mazur

On the basis of results previously reported,[1] it was suggested that at least three factors operate to inactivate phage T4B during freezing and thawing: (1) The phase change from the liquid to the frozen state is sufficient to destroy about half the infectivity of the phage population. (2) Osmotic shock contributes to the injury of rapidly warmed phage, because rapid warming is equivalent to rapid dilution of phage suspended in a concentrated solution. (3) An event occurs during rapid cooling which sensitizes the phage to subsequent inactivation during slow warming by some phenomenon such as recrystallization. Experiments have now been conducted to test the first two of these suggestions and also to attempt to determine the presence or absence of water within the phage particle, and the state and role of this water, if present.

Phages T4B and an osmotic-shock-resistant mutant, T4BO$_1$, were suspended in distilled water or in various solutions and were subjected to a variety of cooling-warming treatments in the range of −2 to −78°C for times from 2 to 50 min. Survival was assayed by standard methods. Some results are shown in Fig. 12.2.1. From these results it can be concluded: (1) No damage occurs at subzero temperatures in the absence of ice formation. (2) The damage accompanying rapid warming from the frozen state is due to osmotic shock. In the first place, the osmotic-shock-resistant mutant, T4BO$_1$, is much less sensitive to rapid warming than is the osmotic-shock-sensitive form, T4B (Fig. 12.2.1*a*). Second, when T4B and T4BO$_1$ are subjected to slow warming, a treatment that eliminates osmotic shock, the survival of the two strains is parallel (Fig. 12.2.1*b*).

We suggest that rapid warming produces an osmotic shock because any solute present would be concentrated during freezing and would be rapidly diluted by rapid warming. Additional results support this view. First, exposure from 10 to 50 min at −10, −50, or −78°C had little or no effect on survival of T4B that had been frozen rapidly and warmed rapidly. However, increasing lengths of exposure at −20 and −30°C resulted in a gradual but continuous decrease in survival of T4B. It is at these latter temperatures that the concentration of NaCl, undoubtedly carried over from the storage medium into the suspending medium, would become maximal.[2] Second, when phage T4B suspended in 0.006 osmolar solutions of KNO_3, NaCl, or $CaCl_2$ was rapidly cooled to and rapidly warmed from temperatures between −20 to −78°C, it was observed that the lower the eutectic point, the lower the survival. For example, after freezing to −78°C, the percent survivals of T4B were: 57% in KNO_3 (eutectic point, −3°C); 44% in NaCl (eutectic point, −21°C); 10% in $CaCl_2$ (eutectic point, −55°C). The lower the eutectic point, the more time there would be for salts to diffuse into the phage head and produce osmotic shock upon rapid warming.

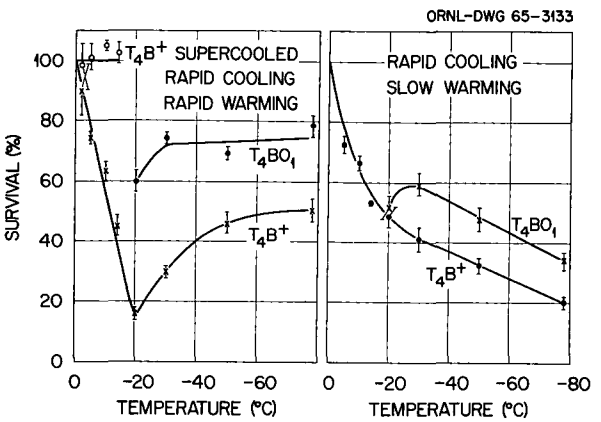

Fig. 12.2.1. Survival of Phages T4B and T4BO$_1$ as a Function of the Minimum Subzero Temperature of Exposure. Phages were suspended in distilled water, and subjected to each of the indicated treatments.

The results of these and other experiments[3] not only support the original suggestions regarding the mechanism of destruction of T4B by freezing but also imply that there is some water with solvent properties present within the phage head.

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12.3 KINETICS OF THE ULTRAVIOLET-INDUCED DIMERIZATION OF THYMINE IN FROZEN SOLUTION

Walter Füchtbauer Peter Mazur

Ultraviolet irradiation of solutions of thymine produces dimers only when the solution is frozen. However, only rough estimates of the quantum yield are available.[1] The present experiments were performed in an attempt to obtain a better estimate and to measure the activation energy for the process. The measurements were made in the following way. A 1-mm layer of thymine solution in a special quartz cell was frozen in an ethanol bath at –10°C, transferred to a quartz Dewar equipped with quartz windows and precooled to the desired temperatures, irradiated for a short time (10 sec to 1 min), and thawed. The amount of thymine converted to dimer was measured spectrophotometrically. Fresh samples were then successively subjected to the same treatment, except they were exposed to increasingly longer irradiation. In this way a curve (Fig. 12.3.1) was constructed, showing the rate of disappearance of thymine (or appearance of dimer). We had hoped to follow the kinetics of a single sample while it was frozen, but this proved difficult because of the clumping effect discussed previously.[2]

The quantum yield was estimated from the rate of dimerization at time 0. This procedure avoided the necessity of correcting for the rate of the back reaction, that is, the splitting of dimers by the same irradiation. It also avoided the necessity of correcting for the fact that, as dimerization proceeds, the chance of a photon hitting an undimerized thymine molecule becomes progressively less.

On the basis of preliminary experiments, the calculated quantum yield (uncorrected for scattering) is about 1.5, compared to Wang's value of 0.2.[1] If the true value is 1, one dimer is produced for every two photons absorbed; if the true value is 2, one dimer is produced each time a single photon is absorbed by thymine. A large factor in the uncertainty as to the correct value is the problem of scattering. Photometric measurements of frozen layers of pure ice show that about 50% of the incident photons are scattered (i.e., fail to reach the photocell detector). The question concerning the frozen thymine solution is how much of this scattered light is absorbed by the thymine and how much escapes from the cell.

Although there is still uncertainty as to the correct value of the quantum yield, the value is high and the dimerization is efficient. In fact, it appears to be considerably more efficient than the dimerization of adjacent thymines in DNA and in dinucleotides in unfrozen solutions. Reported quantum yields in these cases are about 0.03 and 0.01 respectively.[3,4] The high quantum

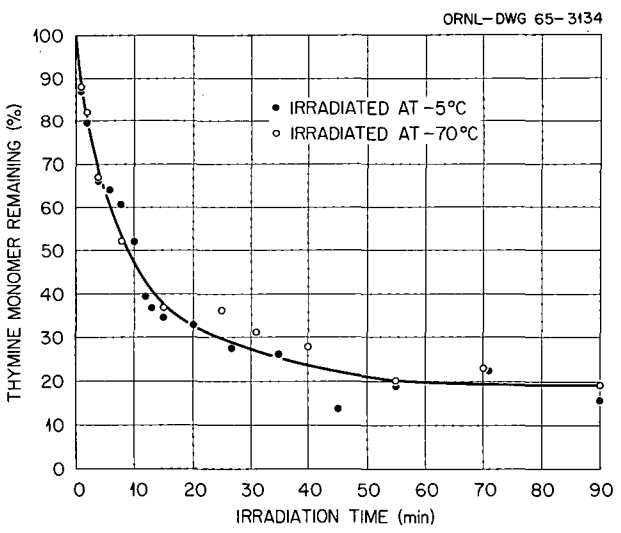

Fig. 12.3.1. Kinetics of Dimerization of Thymine in Frozen Solution During Irradiation at 2537 A. Samples were initially frozen at –10°C and then irradiated at –5°C or –70°C. Each point represents a separate sample.

yield in frozen solution suggests that freezing forces the thymine into specific orientations that are highly favorable for dimerization. This would imply the existence of some interaction between the thymine orientation and the structure of ice.

A final point on the data in Fig. 12.3.1 is that the rate of dimerization appears identical at –5°C and at –70°C. This identity indicates that the activation energy for the process is zero and is, of course, consistent with dimerization being a purely photochemical process.

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12.4 EFFECT OF COOLING AND WARMING VELOCITY ON THE SURVIVAL OF FROZEN AND THAWED YEAST

Peter Mazur Janice J. Schmidt
Rebecca J. Matheson

Introduction. – Two factors are believed to be responsible for lethal injury in yeast cells during freezing and thawing: the concentration of solutes that accompanies progressive solidification of the suspension and the formation of large intracellular ice crystals.[1]

The plot of survival as a function of cooling velocity is complex. If the cells are warmed rapidly at more than 500°C/min, the plot shows two maxima. One is at about 1°C/min; it appears to be the result of the opposite effects of cooling velocity on damage from solute concentration and on damage from intracellular freezing. Cooling velocities much lower than 1°C/min are harmful because they cause the cells to be exposed to concentrating solutes for long periods of time. Cooling velocities above 1°C/min are harmful because intracellular ice formation becomes increasingly probable.[1,2] But when cooling rates exceed about 500°C/min, survival rises once again to reach a second maximum at about 10,000°C/min. This rise is believed to be due to the fact that the higher cooling velocities produce increasingly smaller intracellular crystals and to the fact that small crystals are innocuous. If these conclusions are correct, one would predict that the rate at which the frozen cells are rewarmed will have little effect on cells cooled at 1°C/min but a great effect on cells cooled at high rates. The basis for the latter prediction is that small innocuous intracellular crystals will remain small and innocuous only if warmed rapidly; otherwise, they will grow to damaging size by recrystallization. The reason for the former conclusion is that cells cooled at 1°C/min or less should contain no ice crystals at all. In general, the prediction is that the more the cooling velocity exceeds 1°C/min, the more dependent will survival be on warming velocity.

Experimental. – Suspensions of yeast in water were cooled in a variety of baths and insulated containers to –196°C, at rates varying from 0.2 to 450°C/min, and were warmed at rates ranging from 1 to 1400°C/min. Temperatures were measured with thermocouples and recording potentiometers. Survivals were determined by plate count assay.

Results and Conclusions. – The results are summarized in Fig. 12.4.1 as a log-log plot of survival as a function of warming velocity. The dashed curve shows the previously reported results for cells cooled at about 10,000°C/min in rotating liquid nitrogen.[3] Two main conclusions are evident: (1) Survival increases with increasing warming rate. (2) The less rapidly the cells are cooled, the less the effect of the rate of warming.

Two other features of the data are also of interest.

1. Although the warming rate had a marked effect on cells cooled rapidly at 200 to 500°C/min and although the highest survivals were obtained with the highest rates of warming, the maximum survival was only about 0.02%. In contrast, when the cells were cooled ultrarapidly at 10,000°C/min, the highest survivals were above 1%. Our interpretation of the difference is the following. A cooling velocity of 500°C/min, for example, is high enough to produce intracellular ice in all cells, but it is too low to produce small innocuous crystals except in a very small fraction of the cells. Instead, large intracellular crystals are produced and are lethal, regardless of warming rate.

2. The slowest warming rate of 1°C/min appears to be somewhat harmful when cooling is slow. The extent of injury, however, was found to be dependent on the lowest temperature to which the cells were cooled. When cells were cooled at 1°C/min to −30 or −45°C, slow and rapid warming both yielded equally high survivals. But, when cells were cooled to −60°C or below, as in Fig. 12.4.1, slow warming was considerably more harmful than rapid. The cause of this temperature dependence remains uncertain.

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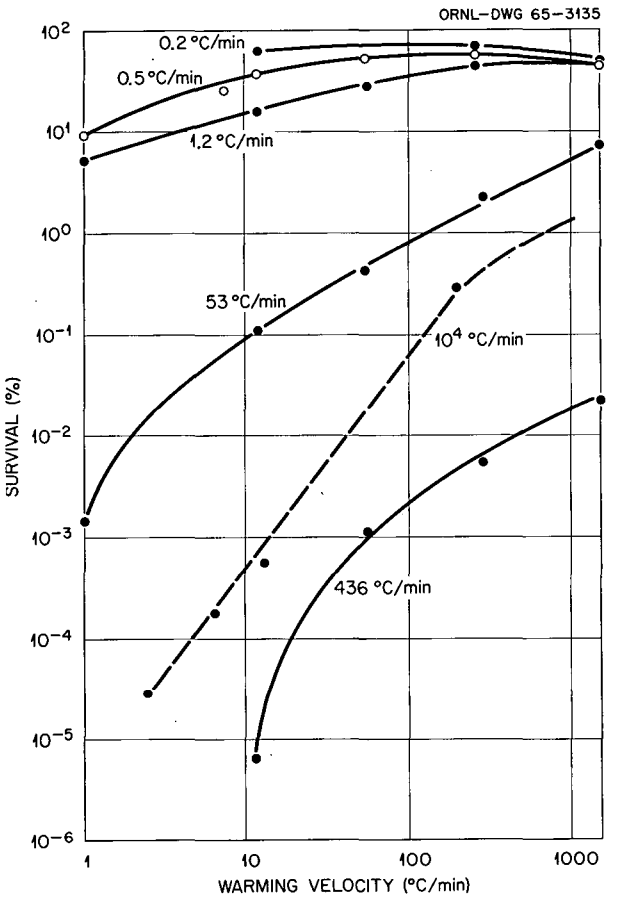

Fig. 12.4.1. Survival of Cells of the Yeast *Saccharomyces cerevisiae* as a Function of Cooling Velocity and Warming Velocity. All suspensions were initially cooled to −196°C. The numbers on the curves are cooling velocities.

13. Chemical Co-Carcinogenesis

(Cooperative Program with NIH-NCI)		Inhalation, Chemical, and Microbiological Co-Carcinogenesis (13.6–13.9)	
Co-Carcinogenesis Basic Program (13.1–13.5)		D. G. Doherty[a]	T. E. Banker
Stanfield Rogers	Barbara R. Bussell[a]	A. P. Harrison, Jr.	J. G. Farrelly
H. I. Adler[a]	D. S. Carroll[a]	M. G. Hanna, Jr.[f]	Anna S. Hammons
E. H. Y. Chu[a]	Frances E. Hacker[a]	Alfred Hellman[g]	F. J. Lacina
C. C Congdon[a]	Joseph Kendrick[a]	R. F. Newell[g]	Minnie L. Lott
G. E. Cosgrove[a]	R. R. Long	Vu-Thi-Suu[a,c]	D. H. Martin
F. J. de Serres[a]	Anita M. Meley[a]		W. W. Miller, Jr.
F. T. Kenney[a]	Peggy H. Thomas		B. G. Moore
R. F. Kimball[a]			R. J. Satterfield
H. V. Malling[a]			J. R. Webb
G. D. Novelli[a]			W. R. Holder, Jr.
Lawrence Rosen[a]			
D. C. Swartzendruber[a]			
A. C. Upton[a]			
G. T. Barry[b]			
Piero Cammarano[a,c]			
A. W. Craig[a,c,d]			
B. J. Kilbey[a,c]			
D. P. Kosow[a,e]			
Marialuisa Melli[a,c]			
Frances K. Patterson[a,f]			
W. D. Wicks[a,e]			

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[c]Visiting investigator from abroad.
[d]British Empire Cancer Campaign of Great Britain.
[e]American Cancer Society Fellow.
[f]Research associate.
[g]Loanee.

13.1 EFFORTS TO TRANSMIT SYNTHETIC GENETIC INFORMATION USING THE SHOPE VIRUS DNA AS A CARRIER

Peggy H. Thomas Stanfield Rogers
R. R. Long

That the Shope virus DNA is infective and produces papillomas identical with those produced with intact virus was reported by Ito in 1960.[1] It seems, therefore, feasible to modify the metabolism of the tumors by incorporating synthetic polynucleotides into the DNA of the virus. As it is evident that the success of this experiment will depend largely upon the number of viral DNA molecules tagged with the polynucleotides chosen, efforts are first being made to determine the conditions of extraction of the DNA from purified virus which give the highest yield of infective DNA. Until now, only DNA extracted from the papilloma has proved infective. Obviously, the stabilizing influence of other components in the extract is important both to the stabilization of the virus DNA in solution during and after extraction and to

other DNA in the extract, which acts to protect the virus DNA from DNase at the time of rabbit inoculation. The influence of these variables has been tested, and we await the results. The experiment is also being done, using DNA from papillomas.

The work of tagging the virus DNA with polynucleotides is to be done in conjunction with Fred Bollum of the Molecular Biology Group.

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13.2 COMPARATIVE STUDIES OF THE STRUCTURE AND MECHANISM OF ACTION OF THE SHOPE VIRUS-INDUCED ARGINASE AND OTHER ARGINASES

Peggy H. Thomas Stanfield Rogers
R. R. Long

Previously reported studies have indicated that the virus-induced arginase differs in all tested physical and chemical parameters from mammalian arginases derived from rabbit dermal appendage tumors, liver, kidney, horse liver, and calf liver.[1] Centrifugal studies strongly suggested that the papilloma arginase is helical in contrast to liver arginase. Another difference is the requirement of manganese activation of liver arginase, not required for papilloma arginase.

Using optical rotatory dispersion and purified enzymes dialyzed overnight against 0.001 *M* phosphate buffer, pH 6.5, it was found that the virus-induced arginase is 40% helical, while either rabbit or calf liver arginase has no detectable helicity. It was then determined to test the effect of manganese activation on liver arginase. After activation by incubation for 3 hr with 0.05 *M* manganese sulfate, liver arginase becomes 10 to 20% helical. The temperature dependence of such activation is indicated by little or no change in helicity upon activation at room temperature. That the ordered structure produced by manganese activation is dependent on the ordered sequence of amino acids making up the molecule and not specifically on the amino acids is indicated by the fact that a hydrolysate of an equal concentration of liver arginase has no demonstrable helicity and acquires none after similar treatment and incubation with manganese. It has also been found that the protein can be fixed with formalin without destroying the helix produced by manganese activities. It should, therefore, be possible to visualize the nonhelical and activated helical form in the electron microscope. This is to be done in conjunction with Walther Stoeckenius of the Rockefeller Institute.

The innate helicity of papilloma arginase, its previously reported small relative number of peptides despite a higher molecular weight (42,000 as opposed to 37,000) than mammalian arginase, its lack of modulation by manganese, and a larger segment of the remainder of the protein molecule suggest that the virus-induced enzyme is rather primitive as opposed to its more sophisticated mammalian counterpart. This is consistent with previously reported evidence indicating that the genetic information for the synthesis of the virus-induced enzyme is viral in origin and is not rabbit information.

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13.3 IS THE SHOPE VIRUS TRANSDUCING RABBIT GENETIC INFORMATION?

Peggy H. Thomas Stanfield Rogers
R. R. Long

There are two lines of Shope papilloma virus – the wild type obtained from the papillomas of field-infected Kansas cottontail rabbits, which induces tumors in domestic rabbits but is not recoverable therefrom, and a virus line found by Shope in such domestic rabbit tumors, which is recoverable in domestic rabbits and is known as the recoverable line.

In the wild rabbit, the recoverable line obtained from domestic rabbit papillomas induces a threonine aldolase. This enzyme is not induced with wild-type virus. In the domestic rabbit, a factor enhancing serine utilization is induced by wild-type virus but not by the domestic-rabbit recoverable line. Upon passage through the wild rabbit,

the domestic recoverable line reverts to wild type[1] about three-fourths of the time on first passage.[2] Associated with this reversion is the loss of the ability to induce threonine aldolase in wild-rabbit epithelium and the acquisition of the factor enhancing serine utilization in the domestic rabbit. There is a wild-rabbit antigen in domestic-rabbit papillomas induced with purified wild-type virus and a domestic-rabbit antigen in wild-rabbit tumors induced with the domestic-rabbit recoverable line. Immunologic studies are under way to determine if the threonine aldolase described in wild-rabbit tumors is a domestic-rabbit protein. Hybridization experiments are being set up to find if a messenger RNA extracted from normal wild-rabbit epithelium can be demonstrated to hybridize with purified wild-type virus DNA, using a virus-DNA-impregnated agar column.[3] It is planned also to tag antibodies against wild-rabbit DNA with ^{135}I to see if any reacts with purified virus DNA. Should this prove to be the case, it will be worth while to tag the antibodies with ferritin to see if the reacting site can be demonstrated with the electron microscope.

If, as seems likely, the threonine aldolase is a domestic-rabbit protein, studies of the metabolism of wild-rabbit papillomas induced with DNA obtained from domestic-rabbit papillomas should indicate whether the information for the synthesis of this enzyme is packaged as an integral part of the virus DNA. If it is simply packaged within the protein coat but unattached, it would not be expected to be induced by the DNA preparation from which the protein coat has been removed by phenol.

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13.4 PROTECTION AND RECOVERY FROM CHEMICAL DAMAGE

C. C Congdon D. G. Doherty
Frances Hacker

Introduction. – This phase of the Co-Carcinogenesis Program is intended to determine if the possibility of protection and recovery from the acute chemical toxicity of carcinogens exists. One other experiment, not yet completed, has been set up to see if AET will prevent lung tumor induction by nitrogen mustard. Other themes of the program, now only secondary, are the development of the experimental pathology of acute chemical injury by carcinogens and the application of the work to transplantation of stem cells that have been treated with carcinogens.

Results and Discussion. – The protection and recovery studies have shown some evidence that bone marrow transplantation can reverse marrow damage by dimethylbenzanthracene.[1] Most of our work has gone into determining whether or not AET will prevent mortality from acute lethal exposures to chemical carcinogens. Some evidence was obtained that AET reduced the mortality from dimethylnitrosamine, but the result has not been reproducible.

In working with AET, glutathione has frequently been used to prevent the toxicity of AET, as reported in 1963.[2] The remarkable ability of glutathione to allow mice that received 20 mg of AET, orally, to survive has been repeatedly confirmed. This finding suggests that one might try giving glutathione with the toxic doses of chemical carcinogens to see if it would reduce their toxicity. Studies of this nature will be undertaken in the next report period.

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13.5 CYTOLOGICAL EFFECTS OF PHLEOMYCIN

Ellen Mattingly

The antibiotic phleomycin has been shown to inhibit DNA synthesis but not synthesis of RNA or protein in both *E. coli* and Hela cells.[1] This inhibition is the result of inhibition of DNA polymerase due to binding of the antibiotic with the DNA primer.[2] In addition, phleomycin has been shown to have some exonuclease activity. This report will consider the cytological effects of this drug in the broad bean.

Lateral roots of *Vicia faba* were treated continuously with a solution of phleomycin (100 μg/ml). At this concentration, the mitotic index dropped very rapidly and after 9 hr of treatment, no divisions were seen. Roots maintained in this concentration of phleomycin showed no evidence of recovery, nor did recovery occur if roots were transferred back to spring water. Experiments using ^{3}H-thymidine showed that, after only 3 hr of treatment, DNA synthesis was completely stopped.

Smears of root tips treated for 3 hr showed numerous divisions still. Cells in prophase appeared quite normal. On the other hand, most metaphase chromosomes appeared very sticky. This condition continued into anaphase, resulting at best in anaphase bridges and, more generally, in numerous micronuclei in each cell. Experiments in which roots were pretreated for 1 hr with ^{3}H-thymidine and subsequently grown in phleomycin showed that the aberrant metaphases were all in G_2 at the time of treatment, and, in fact, no labeled division figures were ever seen.

Studies using lower concentrations (10 μg/ml) produce qualitatively the same results. It was found, however, that roots treated for even 24 hr at this concentration could recover if returned to spring water. At the end of 48 hr of recovery, metaphase cells were numerous and aberrations were frequently seen. Furthermore, interphase cells showed large numbers of micronuclei.

We can conclude from the above results that phleomycin is effective in *Vicia faba* as an inhibitor of DNA synthesis. The observation of an effect on cells in late G_2 supports previous work from this laboratory on FUdR,[3] another DNA synthesis inhibitor, in which it was shown that the shattered chromosomes produced by FUdR are, in fact, produced in G_2 cells. Finally, it is possible that the stickiness produced in the G_2 chromosomes by phleomycin may be the result of the exonuclease activity possessed by the drug.

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13.6 INHALATION CO-CARCINOGENESIS

D. G. Doherty
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R. F. Newell
J. G. Farrelly
Anna S. Hammons
F. J. Lacina
D. H. Martin

The initial construction phase of the Inhalation Program[1] has been recently completed, with the installation and calibration of the x-ray facilities. The aerosol chamber instrumentation and control systems, after minor modifications, have been operated successfully for several weeks, both empty and with a full complement of mice. The apparatus for the creation and control of the gasoline smog and for dust injection has recently been installed and is currently undergoing shakedown.

The experimental design and nature of the Inhalation Program requires animals which are free of mouse pathogens, internal and external parasites, and as many of the murine viruses as is possible, so that the effects of the agents to be used may be properly evaluated. The inability to obtain such animals from commercial sources on a regular basis has prompted us to establish a specific-pathogen-free (SPF) breeding colony. The initial stock was generously supplied by H. E. Walburg, Jr. The mice were offspring of a genetically C57BL/6J stock that was brother-sister mated for four generations, rederived by Caesarean section into a germ-free operation, and foster nursed by ICR germ-free females. This rederived stock was then transferred to our SPF isolation facility, and the colony was started in December 1964, with 37 brother-sister mating pairs. The growth rate of the colony indicates that the foundation stock of 150 breeding pairs will be reached in early March 1965. This will provide a source of approximately 1200 research animals per month. Virus profiles of these animals indicate that they are free of latent mouse viruses. The animals have also been tested and have been found to be free of internal and external parasites, as well as *Pseudomonas sp.* and *Salmonella sp.* An IBM Data Collecting System has been adapted to this colony and has been found to be extremely adequate.

A basic aspect of the Inhalation Co-Carcinogenesis Program is the study of the initiating effect of low-dosage irradiation, in combination with chemical and viral agents, in tumor induction. It is necessary to find a dosage of x irradiation which is low enough to give no deaths and adequate re-

covery but high enough to be effective in successful carcinogenesis.

Preliminary studies on 6-week-old C57BL/6J mice indicate that at a dose rate of 100 r/min the LD_{50} is 654.5 r for males and 647.0 r for females, with a combined LD_{50} of 650.5 r. Thus, two dose levels, 100 r and 200 r, were selected for study of the hematologic and histologic recovery over a 30-day period. This study, although not completed, shows that a dosage of 200 r of x irradiation to 6-week-old mice results in an 88% decrease in the peripheral white blood cell count by 24 hr postirradiation. The recovery, although slower in the males compared to females, was essentially complete by 30 days postirradiation. No deaths occurred in this group of animals. No significant change was observed in the peripheral red cell count, hematocrit levels, or body weights of these animals. Histologically, hypoplasia of the spleen, lymph nodes, and thymus was observed during the early postirradiation intervals. Except for some karyorrhexis in the lymphatic regions, no marked cellular damage was observed in the epithelium of the upper and lower respiratory tract. The animals receiving 100 r of x irradiation had a less severe decrease in peripheral white blood cells at 24 hr postirradiation (63%), with a total recovery at 18 days postirradiation. The histology of this group has not been completed.

Preliminary work has also begun with the other initiating agent, viral infection. A PR8 strain of influenza virus, originally passed since 1934 in Webster strain mice, has been obtained, cultured, and adapted to C57BL/6J mice. This virus, designated A/PR8 34-60, has yielded an LD_{50} of $1 \times 10^{-6.2}$ in the C57BL/6J mouse. We are currently evaluating methods of aerosolizing this virus preparation so that large numbers of animals can be inoculated simultaneously, rather than using the intranasal technique, a method not suited to large numbers of animals. This virus strain is also being used to familiarize the laboratory personnel with the hemoglobin agglutination, hemoglobin agglutination inhibition, and complement fixation reactions. It is planned, in the near future, to replace the A/PR8 34-60 virus with an A_2 influenza strain known to be free of any presently recognizable tumor virus. As soon as "clean" animals are available from the SPF colony, the LD_{50} will be determined, using both the intranasal and aerosol methods of inoculation. In addition, large quantities of A_2 virus will be prepared and preserved for use in the inhalation studies. Methods for purification of the influenza virus, as well as rapid methods for viral isolation and identification, based on experience with polio systems, are being studied in order to provide as pure a virus as possible for experimental use.

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13.7 TIME RELATIONSHIP OF ACTINOMYCIN D AND ANTIGEN TO THE IMMUNE RESPONSE

M. G. Hanna, Jr. C. J. Wust[1]

Introduction. – We have reported[2] that actinomycin D, given at the same time as antigen (sheep erythrocytes) in the rat, caused a delay in the time of appearance of circulating hemagglutinin but had no effect on the apparent rate of synthesis or the maximal amount of antibody eventually produced at five to six days. In the mouse, cellular damage, particularly in the germinal centers of lymphoid tissue, was most pronounced when the antibiotic was given with antigen.[3] Coincident with the cytotoxicity was a delay in the appearance of circulating antibody. As restitution and repopulation of the lymphoid tissues occurred, hemagglutinin was titrated in the serum.

The present study was undertaken to investigate further the time relationship of injection of actinomycin D and antigen in both the rat and mouse.

Methods and Results. *Response in Rats.* – In preliminary studies, 75 μg of the antibiotic given at the same time as antigen (1 ml of 10% sheep erythrocytes) produced maximal inhibition compared to that produced when the antigen was injected at various hourly intervals after actinomycin D. Thus, a second set of animals was treated with a lower dose of actinomycin to determine whether the drug would be more effective if it were given before the injection of antigen. Fifty micrograms of antibiotic was given to each animal in groups of four at 4, 8, 12, 16, 20, 24, or 48 hr before the antigen. Based on the titers found on the fourth day after injection of antigen, maximal inhibition

was found to be 8 hr before the antigen. The relative delay, as measured by the difference in slopes of the responses extrapolated to the base line, was 60 hr. A minimum difference in titer, indicating little, if any, delay in the immune response, was noted when the drug was given 20, 24, or 48 hr prior to the injection of SRBC.

Response in Mice. – Different groups of mice were injected intraperitoneally with 12 μg of the antibiotic 12, 8, and 4 hr prior to the injection of sheep erythrocyte antigen, at the same time as antigen, and 4 and 8 hr after the injection of antigen. Four animals from each group were killed at 6 and 12 hr and 1, 1.5, 2, 3, 4, 5, and 6 days after injection of SRBC. Hemagglutinin titers taken from animals killed between days 2 and 6, after injection of SRBC, are presented in Fig. 13.7.1. The slopes of the curves, indicative of the rate of synthesis of hemagglutinin, are the same when the drug is given before the antigen. This slope tends to be decreased when the drug is given simultaneously or 4 or 8 hr after the antigen; however, since the points are based on pooled sera, no significance can be applied to the difference in slopes. All groups treated with actinomycin displayed a delayed latent period and the same maximum titers as the untreated animals receiving antigen.

Karyorrhexis was a consistent histologic observation in the spleen white pulp of the antigen-stimulated mice treated with actinomycin. During the early intervals (6, 12, and 24 hr after antigen) in the mice receiving actinomycin prior to the antigen, large macrophages containing considerable nuclear debris were located primarily in the spleen-white-pulp germinal centers and scattered throughout the associated lymphocyte mass. By day 2, the lymphatic nodules were atrophic and the germinal centers were depleted of their characteristic cells.

The mice that received actinomycin simultaneously with or after the antigen exhibited the same pattern of histologic changes; however, the severity of damage and cell loss was less pronounced during the early intervals. In general, the degree of damage was directly correlated with the relative delay in hemagglutinin titer. This correlation was found during recovery as well. Repopulation of the spleen-white-pulp germinal centers by the characteristic large basophilic cells was seen in all animals six days after antigen. Morphologic counterparts in nonimmunized animals receiving actinomycin only were not severely damaged by the drug.

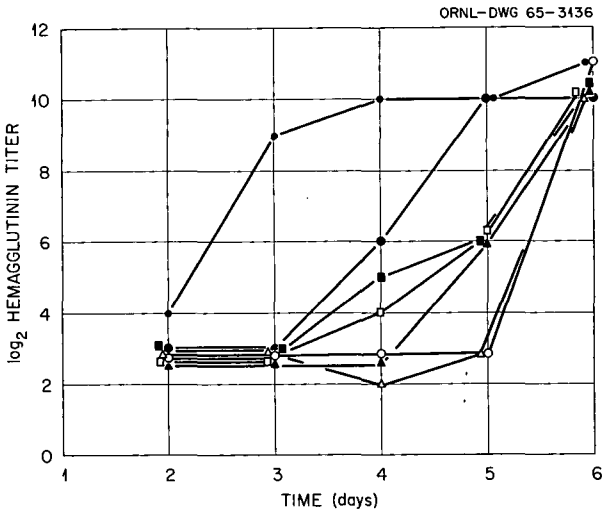

Fig. 13.7.1. The Effect of Actinomycin D on Hemagglutinin Titer in LAF_1 Mice. Actinomycin D injected: ● – ●, 12 hr before SRBC; O – O, 8 hr before SRBC; Δ – Δ, 4 hr before SRBC; ▲ – ▲, simultaneous with SRBC; □ – □, 4 hr after SRBC; ■ – ■, 8 hr after SRBC; ● – ●, SRBC only.

Conclusions. – The present results indicate that the large basophilic cells implicated in the induction phase of the immune response are damaged when actinomycin is given before, with, or after the injection of antigen. A marked correlation is found between the relative delay in the appearance of hemagglutinin and the degree of cellular damage. The most interesting finding is that the severity of the damage is dependent upon the time that the drug is given. The greatest delay and most severe damage is noted when the antibiotic is given 4 to 8 hr before the antigen.

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13.8 BENZPYRENE UPTAKE BY BACTERIA AND YEAST IN ABSENCE OF MUTATION

B. G. Moore A. P. Harrison, Jr.

The present report continues the search for suitable microorganisms with which to examine uptake and metabolism of 3,4-benzpyrene. The methods have already been described.[1]

Results. *Benzpyrene Uptake by* Saccharomyces *and Bacterial Strain 100.* – The three strains of *S. cerevisiae* tested were able to take up benzpyrene. The results (Table 13.8.1) with one, strain Y-2235, are presented here. The assays in the second column (washed cells) represent the radioactivity not removed by the series of serum-broth and buffer washings. This radioactivity will be referred to as "total uptake," and equilibrates within four days to 5.5% of that originally available [6.2×10^6 counts min^{-1} $(5\ ml)^{-1}$] in the culture medium. Little radioactivity is removed by the cold trichloroacetic acid (TCA); most is removed by the hot ethanol and ethanol-ether extractions. But some is not extractable, and equilibrates within four days to represent 0.85% of the total uptake. Possibly this radioactivity is in a protein fraction. This residual radioactivity will be referred to as "fixed."

The radioactivity extractable from washed cells by hot ethanol is also extractable by a single exposure to 90% phenol at room temperature, a procedure that has been employed by Weidel *et al.*[2] to strip *E. coli* down to its cell membrane.

Colonial or auxotrophic mutants in yeast induced by benzpyrene were never detected.

We were prepared to conclude that most of the hydrocarbon was associated with the cell surface, because over 98% of the radioactivity could be removed with hot ethanol and ether (lipid solvents) or with strong phenol (known to remove surface material). Thus, when the first bacterium (strain 100) observed to take up benzpyrene was noted to form mucoid growth on agar, we were attentive. Subsequent discovery of spontaneous rough colonial variants provided a means to examine the role of the cell surface upon uptake. The uptake by strain 100 (mucoid) during growth is plotted in Fig. 13.8.1. By comparison, the rough variant takes up benzpyrene very poorly.

Benzpyrene Uptake and Metabolism by Escherichia coli *Strain M.* – The triple auxotroph of *E. coli* M was cultivated in 100 ml of minimal medium containing leucine, uracil, thymine, and 30% serum with 25 μg/ml benzpyrene. (Dialyzed serum was used.) Benzpyrene is not inhibitory, and uptake is not dependent on cell division.

Cell extracts were chromatographed with *n*-butanol–pyridine–water. Two spots not discernible with the control extracts were observed in addition to the benzpyrene spot. One was a weakly ultraviolet-absorbing spot (Rf = 0.4), whose radioactivity accounted for 18% of the total; the other was a yellow, fluorescent spot (Rf = 0.06) which contained low radioactivity.

Table 13.8.1. Distribution of Radioactivity in Cells and Extracts of *Saccharomyces cerevisiae* Y-2235

Radioactivity available in 5 ml of culture = 6.2×10^6 counts/min

Days Incubation	Distribution of Radioactivity [counts min^{-1} $(5\ ml)^{-1}$]					
	Washed Cells Suspended in Cold TCA	Cold TCA Extract	Hot Ethanol Extract	Hot Ethanol-Ether Extract	Hot TCA Extract	Extracted Cells Resuspended in Ethanol
1	5.5×10^4	4.6×10^2	4.8×10^4	3.7×10^3	1.2×10^3	8.8×10^2
2	1.0×10^5	2.7×10^2	1.0×10^5	3.8×10^3	8.0×10^1	1.6×10^2
3	4.1×10^5	1.9×10^3	3.8×10^5	2.7×10^4	5.3×10^2	2.0×10^3
4	3.4×10^5	1.6×10^3	3.1×10^5	3.2×10^4	4.3×10^2	2.9×10^3
5	3.4×10^5	2.4×10^3	3.1×10^5	3.0×10^4	2.0×10^2	2.9×10^3

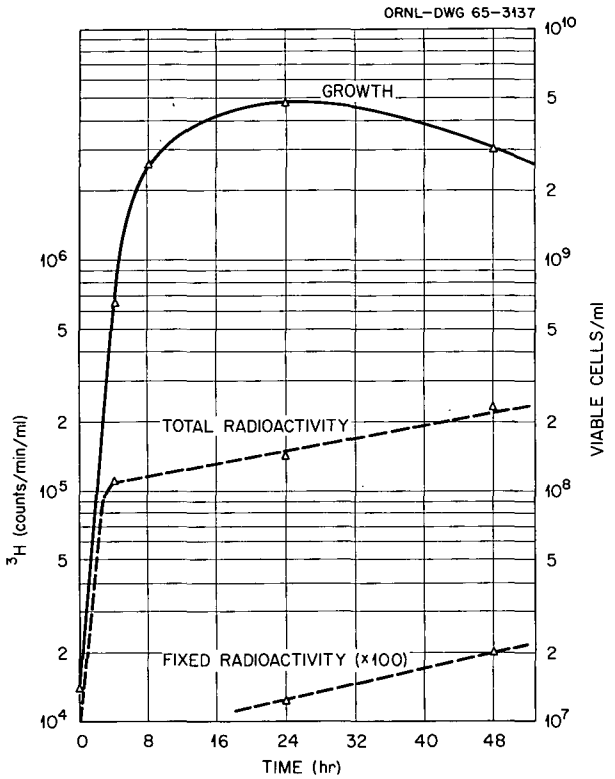

Fig. 13.8.1. Growth with 3,4-Benzpyrene Uptake by Bacterial Strain 100.

Mutagenicity Tests with E. coli *M.* – Table 13.8.2 summarizes one experiment testing for reversion from uracil and thymine dependence (auxotrophy) to independence (prototrophy). From statistical analysis with the thymine revertants, it was concluded with 95% confidence that the difference between control and experimental cultures is less than 35% and with the uracil revertants that there is no difference.[3] Certainly, benzpyrene is not a strong mutagen at these loci. Replicate experiments confirmed this view.

Discussion. – Despite numerous experiments, we detected no mutation induced by benzpyrene, even in those instances in which there was measurable uptake. There was no evidence for benzpyrene incorporation into nucleic acid. Perhaps the use of higher specific radioactivities would demonstrate such incorporation. Although the literature offers little evidence for mutation by benzpyrene,[4] there are reports of interaction between benzpyrene and DNA. For example, De Maeyer and De Maeyer-Guignard[5] observed inhibition of DNA virus (but not RNA viruses) by benzpyrene, and Ts'o and Lu[6] discovered in vitro covalent linkage between thymus DNA and benzpyrene as a result of photoradiation at 320–390 mμ.

Escherichia coli M and yeast should prove useful to study 3,4-benzpyrene uptake and metabolism, especially since parallels exist with mammalian systems. For example, hydrocarbon is fixed to protein, as Somerville and Heidelberger[7] observed with mouse skin homogenates. Further, some metabolism of benzpyrene occurs, and these products may be detected with paper chromatography as employed by Falk *et al.*[8] in their study of benzpyrene metabolism in rats.

Several observations call attention to the role of the cell surface. First, few of the *Escherichia* strains tested were able to take up benzpyrene. Second, with *Escherichia* and other microorganisms, in which uptake did occur, most of the hydrocarbon resided in the cell surface – a fraction extractable by phenol, alcohol, or alcohol-ether. Third, with

Table 13.8.2. Mutation from Auxotrophy to Prototrophy by *Escherichia coli* M when Cultivated With and Without Benzpyrene

	With Benzpyrene	Without Benzpyrene (control)
Total number of cells plated	1.2×10^{10}	3.0×10^{10}
Total number of revertants obtained on uracil-less agar	4.0×10^{1}	1.4×10^{2}
Total number of revertants obtained on thymine-less agar	1.7×10^{2}	3.9×10^{2}
Proportion of the plated cells that were revertants to uracil independence	3.3×10^{-9}	4.7×10^{-9}
Proportion of the plated cells that were revertants to thymine independence	1.4×10^{-8}	1.3×10^{-8}

NOTE: *Escherichia coli* M (triple auxotroph deficient in leucine, uracil, and thymine) was cultivated overnight in the presence of 25 μg/ml benzpyrene and in the absence of benzpyrene (control). Washed cells from the control culture were plated onto 30 synthetic agar plates lacking uracil and onto 30 plates lacking thymine. Washed cells from the benzpyrene-containing culture were plated in like manner. (Benzpyrene uptake was 2×10^{-9} μg/cell.)

the Pseudomonadaceae, it was demonstrated that the loss of this surface material (through smooth-rough variation) greatly diminished benzpyrene uptake. Since the lipopolysaccharide of the cell wall of some bacteria confers upon them their O-antigen specificity, it is tempting to consider that benzpyrene uptake is associated with particular O antigens. This is particularly interesting in relation to the surface phenomena involved in cancer. The Enterobacteriaceae, which have undergone extensive antigenic characterization, might serve as useful models to study the interaction between cell surface chemistry and hydrocarbon uptake. Moreover, mutants are available that cannot synthesize certain polysaccharide precursors.[9]

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13.9 PHYSIOLOGY OF THE VIRUS-HOST INTERACTION

A. Hellman D. H. Martin
J. G. Farrelly

The host-parasite relationship is influenced by the parasite and the host. However, the contribution of each to the degree of pathogenicity for the host is not fully understood. Particularly in the case of viral infection, the contribution of the physiology of the host to a pathological response is not clear. Little is known in the area of hormonal influence on viral infection. Similarly, the hormonal response of the host is intimately related to a variety of environmental stimuli.

In the area of oncogenesis and possible viral induction, the influence of hormones cannot be ruled out, particularly if one considers that the preponderance of malignancies occurs in the 40-year and upper age groups. This age group demonstrates dramatic hormonal changes; consequently, there is some value in investigating the influence of hormones and viral interaction in the area of cancer.

Therefore, we are continuing and extending our previous studies of physioenvironmental factors possibly related to the host-parasite interaction. Having completed a study to determine the response of polio and a host system to hydrocortisone sodium succinate, which indicated that the host response is dependent on time and duration of hormonal stimuli,[1] we are currently extending this study to the influence of insulin and combined hormone stimulus as related to viral induction and synthesis. In this study we are employing both the virulent-avirulent polio system and the influenza system. In the latter case this is an in vivo study. Since the above viral agents are not, feasibly, studied in in vitro and in vivo systems, we are planning to expand this study to the Mengo virus. This agent has several advantages. It is readily cultivated and quantitated in vitro, using cells that are grown on a completely defined medium, thereby making biochemical analyses more feasible. Similarly, it is infective for several rodents, including the mouse, thereby making parallel in vivo studies feasible.

As a corollary to the above, physiostatic studies are also being followed, using an in vitro host system. This study is directed toward determining certain questions as to the host response when the former is maintained at a particular poised state.

Methods for the rapid purification of viruses are being continued, utilizing techniques previously described by us.[2] Similarly, rapid methods of viral isolation and identification are being investigated, utilizing information originally obtained by us, in the polio system.

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14. Subcellular and Viral Physiology

N. G. Anderson[a]

Subcellular and Viral Physiology (14.1–14.3)

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A. A. Barber[b]
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Molecular Physiology (14.4, 14.5)

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14.1 RESPIRATION OF SYNCHRONIZED *TETRAHYMENA PYRIFORMIS*

L. H. Elrod G. M. Padilla

Zeuthen, using single-cell cultures of the ciliate *Tetrahymena*, demonstrated a drop in respiration during cell division.[1] The present study was designed to determine if synchronized *Tetrahymena*, grown in large quantities, conform to the same pattern of respiration.

A parent culture of synchronously dividing *Tetrahymena* was maintained by using the temperature-cycling system described by Padilla and Cameron.[2] Samples from the parent culture were taken at close intervals during the cell cycle and placed in a calibrated 350-ml water-jacketed respirometer in which changes in the partial pressure of oxygen can be measured with a Beckman oxygen electrode. This method allows one to determine the respiratory activity of cells under conditions which are essentially the same as those of the parent culture.

In the first half hour of the warm period, the cells had a Q_{O_2} of approximately 160–180 μl O_2 $(10^6 \text{ cells})^{-1}$ hr^{-1}. This level of respiration remained constant until the onset of the synchronized burst of division. During cell division the respiration rate decreased to 70% of the predivision rate. It further decreased as the cells entered the cold period. These results are in agreement with Zeuthen and may indicate a shift from an oxidative to a glycolytic pattern of energy metabolism.

There are variations in the level and pattern of oxygen utilization of *Tetrahymena* that do not bear a strict temporal relation to the pattern and degree of cell doublings. Changes in other parameters of cellular growth, such as protein, RNA, DNA, and dry weight content of the cells, are being determined to elucidate further their involvement in the energetics and control of cell division as measured by these techniques.

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14.2 A LONG-LIVED RNA IN SYNCHRONIZED *TETRAHYMENA PYRIFORMIS*

G. L. Whitson G. M. Padilla
L. H. Elrod

The purpose of this study is to show that a relatively stable RNA is associated with morphogenesis and cell division in temperature-synchronized *Tetrahymena pyriformis*. These experiments were performed by applying the techniques devised by Levinthal *et al.*[1] for the study of mRNA turnover in bacterial cells. Synchronized cells were sampled just prior to cell division, given a 30-sec exposure to ^{14}C-uracil, and then actinomycin D was added in varying concentrations. Samples were removed from control and experimental cultures at different times, extracted with cold 5% TCA for 30 min, collected and washed on Millipore filters, dried, and then counted with a gas flow counter. The uptake of ^{14}C-uracil was linear with time in the control synchronized cells. On the other hand, the uptake of ^{14}C-uracil in cells treated with actinomycin (20 μg/ml) remained unchanged for 10–15 min, but then gradually increased linearly with time up to 1 hr. With 50 μg/ml of actinomycin, further incorporation of labeled uracil after addition of this antibiotic was not observed, and this label did not decay for at least 1 hr thereafter. Assuming that the initial uptake of ^{14}C-uracil goes into mRNA in *Tetrahymena*, these results indicate that this fraction of RNA is relatively stable and does not have a short half-life as indicated by the rapid turnover (a matter of a few minutes) of mRNA in certain bacterial cells.

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14.3 EFFECTS OF ACTINOMYCIN D ON THE RIBOSOMES OF SYNCHRONIZED *TETRAHYMENA*

G. L. Whitson W. D. Fisher
G. M. Padilla

Introduction. – Evidence for an actinomycin D sensitive period in temperature-synchronized *Tetrahymena* has been reported.[1–3] Actinomycin D (10 μg/ml) added 90 min before the shift from the cold temperature to the warm period (cycle time = 9.5 hr at 12°C and 2.5 hr at 27°C) blocks cell division in more than 90% of the cells. Stomatogenesis is also prevented by this treatment.[3,4] This report presents evidence that the ribosome complement is altered by exposure of these cells to actinomycin D.

Methods. – In these studies, both control synchronized and actinomycin D–treated cells were lysed at different times during the synchronized cycle with saturated indole containing 10^{-2} *M* Tris buffer with 5×10^{-4} *M* Mg^{2+} at pH 7.5. The B-IV zonal ultracentrifuge designed by N. G. Anderson *et al.*[5] and a Beckman model E analytical centrifuge were used for the isolation and analysis of the ribosomes.

Results and Discussion. – An examination of the ribosome profile of cells 1 hr after the beginning of the warm period showed a decrease in the 84S ribosome fraction, an increase in 120S ribosomes, and increases in the larger-size classes of ribosomes. Profiles of cells treated with actinomycin D 1 hr before the shift from the cold to the warm period and then lysed 1 hr after the beginning of the warm period resembled profiles of cells lysed at the end of the warm period (postdivision cells). A further analysis of a portion of the ribosome complement from the model E analytical centrifuge is shown in Table 14.3.1 below. A class of ribosomes with a value of about 70S was

Table 14.3.1. A Comparison of $[s_0]$ Values of Ribosomes from Cells at Different Times During the Synchronized Cycle

End of cold period	82.8		57.2	29.5
Predivision warm period	83.7		61.8	33.1
Postdivision beginning of cold period	82.1	70.0	57.9	29.0
Actinomycin D–treated cells	81.5	71.5	59.3	32.4

observed in both the actinomycin D–treated and the postdivision cells. This similarity between actinomycin D–treated cells with cells which have just finished cell division and which are no longer undergoing morphogenesis is consistent with the view that ribosome changes or a breakdown of polysomes occurs in cells which are not actively engaged in protein synthesis and morphogenesis. Whether or not these changes reflect the removal of protein or the loss of some kind of "informational RNA" at the ribosomal site is unknown.

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14.4 SEPARATION OF ACID-SOLUBLE NUCLEOTIDES AND RELATED COMPOUNDS FROM *TETRAHYMENA* DURING DIFFERENT GROWTH CONDITIONS

W. D. Fisher I. L. Cameron

The chromatographic separation methods described by Anderson *et al.*[1] have been applied to cold acid-soluble extracts of *Tetrahymena pyriformis*. Spectrophotometric analysis of the column effluent at 260 and at 280 mμ shows more than 40 peaks. Comparison of the peak patterns with those of mixtures of known purine and pyrimidine bases, nucleosides, and nucleotides, as well as the 260/280 mμ absorbance ratios, permits identification of the nucleotide derivatives from *Tetrahymena*. Changes in nucleotide content are found during different growth phases.

In the logarithmic growth phase we have tentatively identified 11 of 41 optical density peaks. The number of observed peaks drops to about 26 as the cells reach stationary phase. These studies are continuing and will be expanded to include an analysis of the nucleotide pools of synchronously dividing *Tetrahymena*.

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14.5 ISOLATION OF MACROGLOBULINS BY ZONAL CENTRIFUGATION

W. D. Fisher R. E. Canning
Tom Grizzard

Introduction. – The preparation of highly homogeneous and well-characterized macroglobulins would be useful in understanding the immunological role of macroglobulins. Previously[1] we reported the isolation of an apparently homogeneous rat macroglobulin by separation on a sucrose gradient in the B-IV zonal centrifuge. This report gives some preliminary data on the separation of mouse macroglobulin.

Results. – The macroglobulin from approximately 30 ml of pooled C57 mouse serum was separated on a 1-liter 10–20% sucrose gradient containing a 0.2 μ Miller-Goldner buffer, pH 7.5. The macroglobulin fraction was concentrated by pressure dialysis against buffer, and the pooled macroglobulin from five runs was reprocessed through the same gradient and again concentrated by pressure dialysis. The yield was 8 ml of a 4.95% protein solution. Analytical ultracentrifugation showed a large peak ($[S_0]_{20}$ = 19S) and a small amount of a heavier component ($[S_0]_{20}$ = 30S).

Discussion. – The preliminary results are being extended to include other physical measurements and immunological tests for purity. Additional studies are also needed to determine whether or not the heavier component is an aggregate.

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15. NIH-AEC Zonal Ultracentrifuge Development Program

Basic Studies (15.1, 15.2)	Respiratory Virus and Viral Subunit Isolation (NIAID) (15.3)
N. G. Anderson[a] A. A. Barber[b]	N. G. Anderson[a] P. A. Van Dreal[c] R. E. Canning[a] Margaret T. Guess Charlotte A. Langer[d] G. B. Cline[c]
Tumor Virus Isolation Studies (NCI)	
N. G. Anderson[a] W. D. Fisher[a] Mary L. Barber[c] E. L. Candler, Jr. Tom Grizzard[a] C. T. Rankin, Jr. Elizabeth L. Rutenberg[c]	[a]Dual assignments. [b]Sabbatical leave from the University of California. [c]Consultant. [d]Temporarily at Bethesda, Md.

15.1 ISOLATION OF NATIVE GLYCOGEN BY COMBINED RATE-ZONAL AND ISOPYCNIC CENTRIFUGATION

A. A. Barber W. W. Harris
N. G. Anderson

Introduction. – Glycogen is normally prepared from tissues by extraction with strong solvents. These extraction procedures degrade the native material. Glycogen polydispersity makes it difficult to isolate native material in pure form by differential centrifugation. Zonal and isopycnic centrifugation were combined to isolate fractions of glycogen into particles of uniform size.[1,2]

Results. – Glycogen was rate separated into fractions of uniform particle size by the zonal centrifugation of homogenates in sucrose density gradients. It was then isolated from other cell constituents having similar sedimentation coefficients by isopycnic centrifugation in CsCl to effect a density separation. A density of 1.62 was isopycnic for glycogen. A continuous spectrum of particle sizes up to 200 mμ was observed, and S_{20} values up to 10,000 were measured. A decrease in molecular packing was noted in particles of larger sizes. Microscopically, three levels of glycogen structure were observed, including fundamental units 2.5 to 3.5 mμ in diameter, subclusters 20 to 25 mμ in diameter, and clusters of increasing size up to a maximum of 200 mμ (Fig. 15.1.1). The 2.5-mμ particles were considered to represent the exterior side chains of the branching glycogen structure, and the 20-mμ particles probably represent the particle size normally studied following KOH extraction. The glycogen was isolated free of contaminating membranes.

Discussion. – The isolation of native glycogen into particles of uniform size, free of contaminating membranes, is very important for studies of enzyme binding to glycogen particles and for establishing the existence of free and fixed glycogen fractions in tissues. Rate-zonal centrifugation combined with isopycnic banding provides a method for isolating large quantities of uncontaminated glycogen in fractions of uniform particle sizes for these and other studies on the biochemistry of native glycogen.

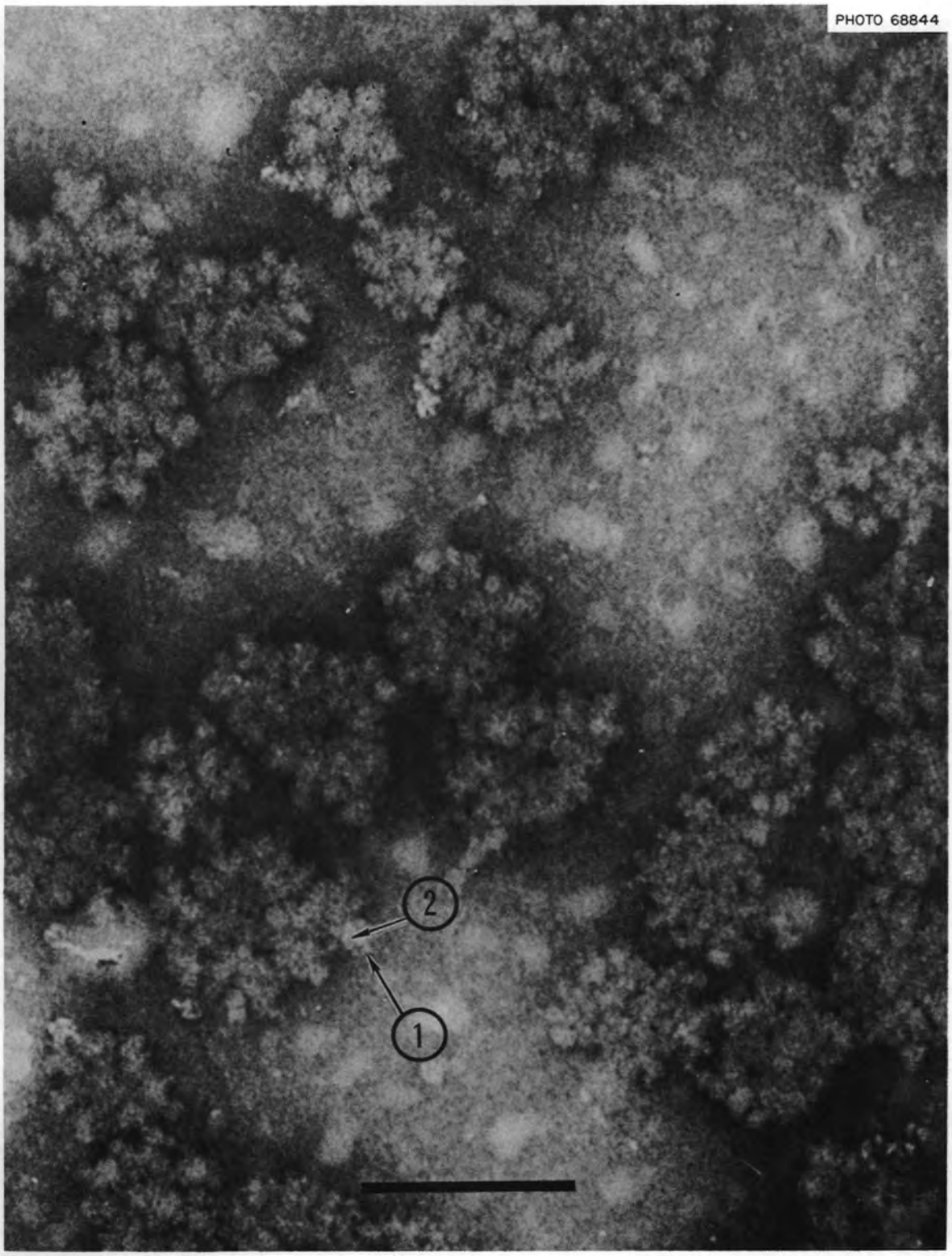

Fig. 15.1.1. Electron Photomicrograph of Negatively Stained Glycogen Prepared by Rate-Zonal Followed by Isopycnic Centrifugation. 1 and 2 refer to the units and subclusters of glycogen respectively. Magnification marker – 0.1 mμ.

References

[1]N. G. Anderson and C. L. Burger, *Science* **136**, 646–48 (1962).

[2]W. D. Fisher, G. B. Cline, and N. G. Anderson, *Anal. Biochem.* **9**, 477–82 (1964).

15.2 LIPID PEROXIDATION IN RAT TISSUE PARTICULATES SEPARATED BY ZONAL CENTRIFUGATION

A. A. Barber C. T. Rankin, Jr.
N. G. Anderson

Introduction. – The development of the zonal centrifuge has made it possible to separate tissue particulate fractions with increased resolution.[1] The present study was designed to separate tissue fractions in a sucrose gradient and to determine the extent of lipid peroxidation in all fractions by aerobically incubating similar volumes of each with ascorbic acid. The aim was to establish peroxidation in mitochondria prepared free of microsomes.

Results. – The distribution of total protein, particulate protein, and lipid peroxidation activity in homogenate fractions of rat liver, brain, kidney, and testis was determined. The results of these determinations on liver homogenates are summarized in Fig. 15.2.1. Lipid peroxidation occurred in all particulates, but the amount of peroxidation differed in different size particulates from the same tissue as well as in similar size particulates from different tissues. For example, peroxidation in microsomal suspensions was greater than that in mitochondria, but liver microsomal activity was much greater than that of testis microsomes. Sonication of mitochondrial fractions demonstrated that the extent of peroxidation was not due to size alone and was presumed to be due to specific differences in lipid and iron content. The particle profiles from different tissues were different, so the procedures designed for the differential centrifugation of one are not applicable to others. Rate-zonal centrifugation with continuous monitoring of the collected gradient provides an efficient method for establishing the particle profiles of tissues on which to base specific procedures of differential centrifugation.

Discussion. – All tissue particulate fractions from liver, brain, kidney, and testis undergo lipid peroxidation when aerobically incubated with ascorbic acid. The peroxidation of mitochondria lipids in the absence of microsomes is of major significance, since lipid peroxidation has been implicated as a mechanism of mitochondrial swelling.[2] The isolation of mitochondria free of microsomes is difficult by the differential methods of centrifugation. Zonal centrifugation provides an effective method for isolation of membranes into fractions on the basis of size.

References

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15.3 MYXOVIRUS AND RHINOVIRUS ISOLATION BY CONTINUOUS-FLOW CENTRIFUGATION

G. B. Cline N. G. Anderson

Introduction. – Certain viruses which cause mild upper respiratory difficulties in adults are known to cause severe lower respiratory tract difficulties in children and young infants.[1] In collaboration with the Laboratory of Infectious Diseases of the National Institute of Allergies and Infectious Diseases, NIH, attempts have been made to isolate and purify two of these viruses, respiratory syncytial (RS) and ECHO 28, by continuous-flow centrifugation. One technique has been found which permits the rapid isolation and concentration of infectious RS particles from noninfectious but antigenically active viral subunits. Preliminary studies on the rhinovirus ECHO 28 indicate that it too can be rapidly and easily isolated from large volumes of harvest fluid by continuous-flow centrifugation.

Results and Discussion. – Studies on myxoviruses have shown that infectivity is greatly reduced when the particles are pelleted by centrifugation or subjected to dialysis.[2] These characteristics limit the methods available for concentrating RS virus. The B-IX continuous-flow rotor has been used in the studies reported here

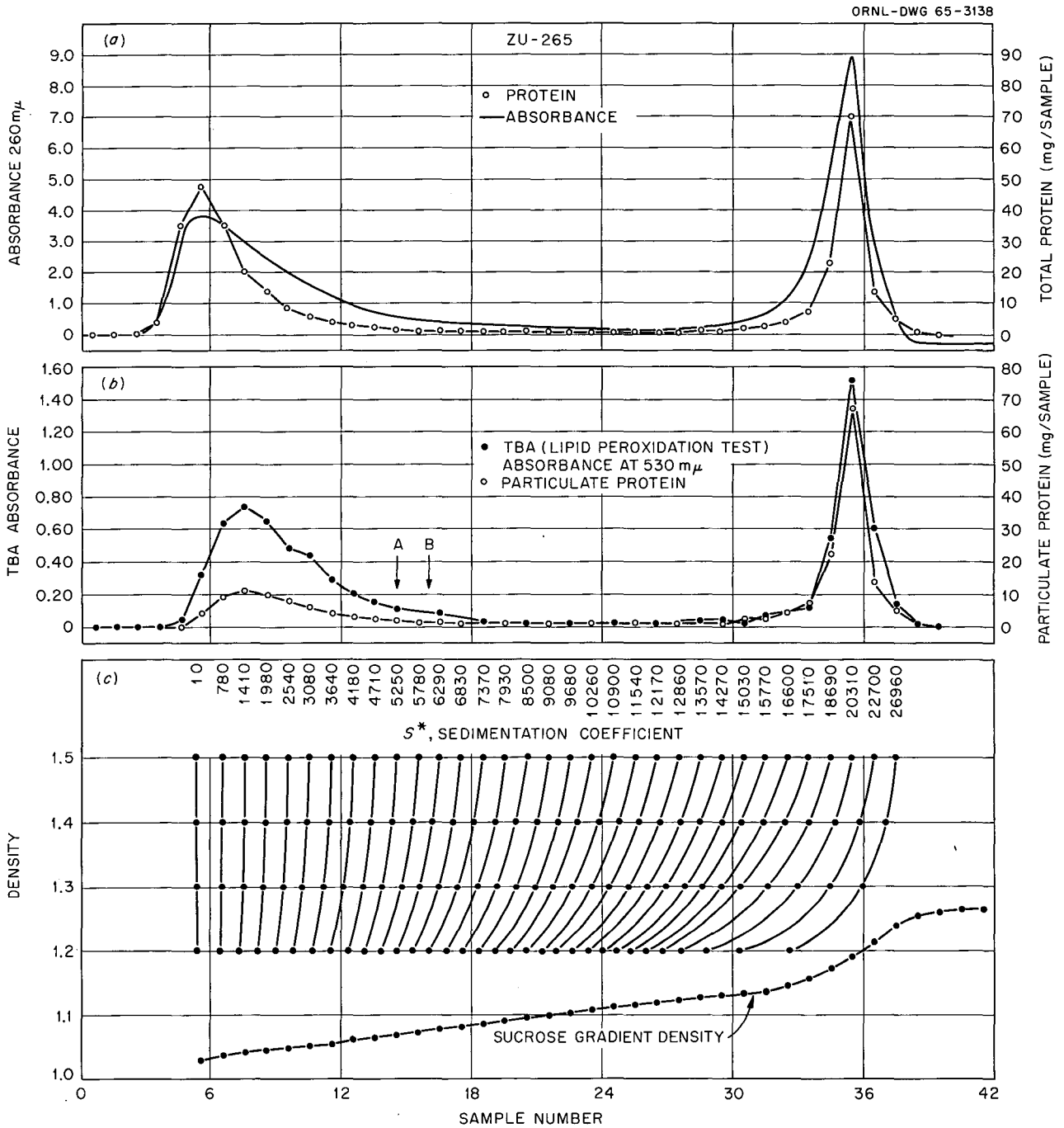

Fig. 15.2.1. Distribution of Ultraviolet Absorbance, Total Protein, Particulate Protein, and Lipid Peroxidation Activity in a Rat Liver Homogenate Centrifuged 10,000 rpm for 15 min in the B-IV Rotor. Twenty milliliters of homogenate (1:10 w/v) was used. Forty-two samples of 40 ml each were collected, and the particles were sedimented. Lipid peroxidation was carried out on 2 ml of resuspended pellets. Explanation of A and B in *b*: The microsomal pellet prepared by centrifuging a 10,000-rpm 15-min supernatant (Spinco No. 30 rotor) at 40,000 rpm for 60 min contains 14% of the liver protein. The corresponding 14% fraction obtained by zonal centrifugation is the particulate fraction to the left of point A. This point is similar to point B, which is the theoretically determined value of 7500S calculated for membrane particles sedimented at 10,000 rpm for 15 min in the Spinco No. 30 rotor.

to band and collect RS virus from liter volumes of harvest fluid in sucrose density gradients. Infectivity has been retained by making the density gradients slightly hypertonic with the addition of phosphate buffered saline (PBS), pH 7.0. Human serum (1.25 mg/ml) has been added to the gradient material to help maintain the integrity of the viral particles. Under these conditions, better than 99.9% of the infectious particles have been cleared from solution at a flow-through rate of 2 liters/hr at a rotor speed of 30,000 rpm. Latest experiments indicate that infectivity can be increased 3 log units over the harvest fluid.

At flow rates where better than 99% of the infectious RS particles are trapped by the B-IX rotor, few of the viral subunits are retained by the sucrose gradient. The viral subunits can be collected from the B-IX effluent by continuous flow through the B-V rotor. The viral subunits have sedimentation rates of 17S and 5S, while the infectious particles have a range of sedimentation rates from 250S to an estimated 2500S. The infectious particles band in sucrose at a density of 1.15 to 1.17, and band in sucrose-PBS at a density of 1.17 to 1.19. The subunits containing the complement-fixing (CF) antigen band at still a higher density than the infectious particle.

Electron microscopy of particles collected by the B-IX rotor shows the presence of particles closely resembling the measles virus described by Waterson *et al.*[3] However, crude particle counting in the electron microscope indicates fewer intact particles than detected by infectivity assay. The disparity may be particle disruption due to the addition of phosphotungstic acid in preparation of the grids.

Respiratory syncytial subunits can be collected from the B-IX effluent by precipitation with ammonium sulfate. Maximal CF activity is precipitated at 60% saturation. However, under these conditions, serum proteins from the harvest fluid are also precipitated.

Preliminary studies on centrifugal techniques of concentrating and purifying the rhinovirus, ECHO 28, indicate that it is trapped by the B-V continuous-flow rotor at flow rates predicted by sedimentation theory.[4] Viral preparations from recent studies in collaboration with Abbott Pharmaceutical Company[5] are now being assayed to determine more precisely the optimal conditions for isolation of ECHO 28 virus from large quantities of harvest fluid. Like RS virus, the low virus yield of ECHO 28 in harvest fluids (10^5/ml) has been a serious limitation in obtaining sufficient virus material and viral subunits for biochemical studies and for vaccine production.

Studies are continuing to establish an efficient technique of isolating and purifying myxoviruses, in general, and respiratory syncytial virus and viral subunits, in particular, from liter quantities of harvest fluids. A one-step continuous-flow centrifugation method is desirable where infectious viruses and viral subunits are recovered in two separate fractions from the gradient material. Studies are now in progress on methods of breaking infectious RS particles into antigenically active subunits with detergents and detergent-ether treatments and with osmotic shock.

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[5]Abbott Pharmaceutical Co., North Chicago, Ill.

16. Nucleic Acid Chemistry

Nucleotide Chemistry (16.1)		Molecular Biology (16.2–16.4)	
W. E. Cohn	A. Jeannine Bandy	F. J. Bollum[a]	Patricia N. Howard
Mayo Uziel		B. K. Zimmerman[a]	Janet C. Ring
		Giovanni Cassani[a,b]	Patricia A. Williams
		J. M. Goncalves[b,c]	

[a]Dual assignments. [b]Visiting investigator from abroad. [c]Fulbright Fellowship.

16.1 ION EXCHANGE CHROMATOGRAPHY OF OLIGONUCLEOTIDES

W. E. Cohn Mayo Uziel
A. Jeannine Bandy

The continued attack on technical problems related to the establishment of composition, sequence, and structure of nucleic acid constituents continues, with particular emphasis on analytical methods of separation, recovery, and analysis. The two areas presently under attack are those of analysis by column chromatography and of recovery of nucleotide material from salt solutions.

Among the problems to be solved in devising rapid, quantitative, and nondegradative procedures for the separation and recovery of nucleic acid components is the removal of the salts used in column chromatography. Certain of the presently routine procedures – for example, those requiring the lyophilization of ammonium bicarbonate – are of questionable utility when applied to complex oligonucleotides or polynucleotides. Accordingly, we have explored methods of desalting that are operable without heating or change in pH.

The copolymer Retardion, an ion-retarding resin, effectively allows the recovery of nucleotide material in water. The selectivity characteristics of this material are unique, however, so that its use is practical only with chlorides and bromides (especially the latter) among common anions, and with the ammonium ion among cations. From 60 to 75% of an oligonucleotide mixture can be completely desalted without discrimination among the species present by passage through a Retardion column; ordinarily this can be done in a matter of minutes. The remainder of the mixture can be recovered either by recycling or by elution with additional salt; the remainder appears, paradoxically, in the salt-free eluant.

Compounds such as urea or bicarbonate, compounds that cannot be handled by Retardion, can be satisfactorily removed from trinucleotides or larger oligonucleotides with Sephadex G-25. At high salt concentrations tri- and tetranucleotides are found in the nonexcluded (salt) fractions. An interesting by-product of this work is the observation that the products of alkaline hydrolysis of RNA can by passage through Sephadex G-25 be quantitatively recovered free of alkali.

One further technical improvement has been made. The Anderson Nucleotide Analyzer has been fitted with a scale expander with which a quantitative mononucleotide analysis can now be made on as little as 0.1 mg of RNA or similar material.

16.2 BIOSYNTHETIC POLYDEOXYNUCLEOTIDES

F. J. Bollum P. A. Williams
J. C. Ring

A large variety of useful products have now been derived or can be readily prepared by the enzymatic procedures developed by this group.[1] A partial list is presented in Table 16.2.1, and the complete list would contain well over 100 oligomeric and polymeric derivatives.

The single-chain polymers are useful for studies on the mechanism of DNA-polymerase and RNA-polymerase reactions. They are also used for the synthesis of polydeoxynucleotides containing two complementary chains. The double-chain polymers are of further use in studies on the mechanism of RNA polymerase and of certain kinds of DNA polymerase. Our primary uses of these polymers have been in studies on the coding problem (polypeptide synthesis[2,3]), and double-chain polymers also provide chemical definition for investigations on radiation damage and repair.[4,5] The oligodeoxynucleotides obtained by degradative synthesis from the polymers are useful for additional biosynthesis and possibly for immunochemical investigations.

We have now completed most of the synthetic phase of this problem, and future research will concern analysis of biological activities and an extension of this synthetic approach.

References

[1]F. J. Bollum *et al.*, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1964,* ORNL-3700, p. 148.

[2]Giovanni Cassani, P. A. Williams, and F. J. Bollum, "Biological Properties of Synthetic Polydeoxynucleotides," this report.

[3]J. L. Epler, "Aminoacyl-sRNA Binding Properties of Biosynthetic Polynucleotides," this report.

[4]J. K. Setlow and M. E. Boling, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1964,* ORNL-3700, p. 38.

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16.3 BIOLOGICAL PROPERTIES OF SYNTHETIC POLYDEOXYNUCLEOTIDES

Giovanni Cassani P. A. Williams
F. J. Bollum

Three biological characteristics of the biosynthetic polydeoxynucleotides prepared by this group are under investigation:

Table 16.2.1. Biosynthetic Polydeoxynucleotides

Terminal Transferase Polymer	Replicative Transferase Polymer	Oligodeoxynucleotides by Degradation
Poly dA[a]	Poly dA:dT	A_m, T_m $m = 2, 3, 4, 5, 6$
Poly dI[a]	Poly dI:dC	I_m, C_m $m = 2, 3, 4, 5, 6$
Poly dC	Poly dG:dC	G_m, C_m $m = 2, 3, 4, 5, 6$
Poly d(A,C)[b]	Poly d(A,C):d(T,G)	AC, CA, CCA, CAC, ACC, AAC, ACA, CAA, ...
Poly d(A,I)	Poly d(A,I):d(T,C)	AI, IA, IIA, IAI, AII, AAI, AIA, IAA, ...
Poly d(I,C)	Poly d(I,C):d(C,G)	IC, CI, CCI, CIC, ICC, IIC, ICI, CII, ...
Poly d(A,C,I)	Poly d(A,C,I):d(T,G,C)	..., ACI, CIA, ICA, CAI, IAC, AIC, ...
Poly d(CAI)[c]	Poly d(CAI):d(GTC)	

[a]General structure X_mA_n or X_mI_n; large families of polymers in this series where X and *m* are independently varied, for example, T_3A_n, T_4A_n, ... ; I_3A_n, I_4A_n, ... ; etc.

[b]The comma denotes "sequence unknown"; a random copolymer.

[c]Absence of comma denotes "sequence known," that is, $d(C_{67}A_{102}I_{111})$ is a block copolymer containing (on the average) a run of 67 C's followed by 102 A's followed by 111 I's.

1. The transcription of chains and sequences in the polynucleotide complexes by RNA polymerase. Our initial results demonstrate that the double-chain complexes are good templates for RNA polymerase, producing poly*ribo*nucleotide products. It has also been observed that asymmetric copying of the polydeoxy chains is related to the base composition of the chains. For example, the TC chain of d(A,I):(T,C) is copied (producing *ribo AG*) at 4 times the rate of the AI chain. Fine structure of the regulation process can be investigated with this approach.

2. The ability of poly*ribo*nucleotides, directed by biosynthetic polydeoxynucleotides, to bind aminoacyl-s RNA to ribosomes. Our poly*ribo*nucleotides are active and are discussed in this report by Epler.[1]

3. The ability of poly*ribo*nucleotides, directed by biosynthetic polydeoxynucleotides, to bring about the synthesis of specific polypeptides. We have found that our synthetic poly-U directs synthesis of polyphenylalanine from phenylalanyl-s RNA, and that poly-A directs synthesis of polylysine. These pilot experiments encourage us to attempt an examination of the fine structure of the decoding process.

References

[1]J. L. Epler, "Aminoacyl-s RNA Binding Properties of Biosynthetic Polynucleotides," this report.

16.4 THE ENZYMATIC SYNTHESIS AND DEGRADATION OF DNA IN *MICROCOCCUS LYSODEIKTICUS*

B. K. Zimmerman

The enzyme DNA polymerase, purified 1000–1500 fold over crude extracts of *M. lysodeikticus*, effects the incorporation of dNTP's into a polydeoxynucleotide chain in the presence of DNA or a synthetic polynucleotide with the liberation of inorganic pyrophosphate (PP_i). Also present in this enzyme preparation is an exonuclease activity that, by steps, degrades DNA from chain ends into 5′-deoxyribonucleoside monophosphates and inorganic phosphate.

The exonuclease requires the presence of a mixture of either all four deoxyribonucleoside triphosphates (dNTP's) or the deoxyribonucleoside diphosphates (dNDP's) for activity, whereas the polymerase activity absolutely requires dNTP's. The dNTP concentration dependence is identical for the polymerase and the exonuclease. A somewhat greater concentration of dNDP's than of dNTP's is required for maximal activity.

Hydrolysis of DNA has also been observed in the presence of high levels of inorganic pyrophosphate. However, the kinetics of this reaction suggest that the initial event is a reversal of the polymerization reaction, followed by stimulation of the exonuclease activity by the nucleotides released. The products of this hydrolysis are primarily 5′-dNMP's.

Attempts are currently being made to separate the polymerase and the exonuclease activities. Also under investigation are new procedures for isolating DNA polymerase, nucleases, and other enzymes that may play a role in cellular DNA synthesis.

17. Nucleic Acid Enzymology

Elliot Volkin

Microbial Systems (17.1–17.7)

Elliot Volkin
M. Helen Jones
W. E. Barnett
R. K. Fujimura
J. X. Khym
J. L. Epler[a]
C. G. Mead

Developmental Biochemistry (17.8–17.10)

F. J. Finamore
Rose P. Feldman
Sumiko Iyeiri[b,c]
A. H. Warner[d]

[a]USPHS Fellow.
[b]Visiting investigator from abroad.
[c]Damon Runyon Memorial Fund.
[d]Research associate.

17.1 BIOCHEMICAL TRANSFORMATION OF BACTERIOPHAGE T_2 DNA

Elliot Volkin M. Helen Jones

Selective labeling techniques using deoxyuridine-^{3}H as a precursor to label T_2 DNA pyrimidines has allowed further insight to the observation that the nucleotide, hydroxymethyldeoxycytidylic acid (HMC), is transferred to progeny T_2 DNA in a lower amount relative to the other three parental deoxynucleotides.

Experimental. – T_2 DNA was labeled exclusively from the host bacterial DNA by the following technique. *Escherichia coli* B was grown to a titer of 4×10^8/ml in the presence of deoxyuridine-^{3}H. Unlabeled uridine was added to prevent labeling of RNA. Thirty minutes prior to the addition of infecting phage a large excess of cold deoxyuridine was added to the culture, and 45 min after infection the labeled phage produced was collected and highly purified. The lysate was treated heavily with pancreatic DNase, then subjected to differential centrifugation.

Another stock of labeled T_2 was prepared in which isotope was incorporated only from DNA newly synthesized from exogenous culture constituents. Unlabeled bacteria were infected with T_2; then 8 min after infection, deoxyuridine-^{3}H (and cold uridine) was added to the culture medium. At this time, bacterial DNA synthesis had ceased. The progeny phage produced 45 min after infection was purified.

These two phage stocks were used in subsequent experiments designed to follow the transfer of the pyrimidine deoxynucleotides from parental DNA to progeny DNA.

Results. – In T_2 labeled from bacterial DNA, the HMC was of much higher specific activity than was thymidylic acid. In the course of transfer of this DNA to progeny, an excess of the HMC was *not* transferred, relative to the transfer of thymidylic acid. Experiments to question the possibility of a contaminant in the parental phage stock were carried out by subjecting the phage to the further purification of sucrose gradient sedimentation and cesium chloride equilibrium centrifugation. There was no change in the specific-activity ratio of HMC/thymidylic acid as a result of these processes.

In the T_2 stock labeled de novo from exogenous deoxyuridine-^{3}H, HMC had a somewhat lower specific activity than thymidylic acid. There was no deviation in the relative transfer of these deoxynucleotides to progeny DNA.

Discussion. – It is hoped that these experiments may offer some explanation for the high degree of fragmentation of T_2 DNA after infection. Related to this question is the known high degree of genetic recombination that occurs in this system.

17.2 PURIFICATION AND CHARACTERIZATION OF THE OLIGODEOXYRIBONUCLEOTIDE TRANSFERASE SYSTEM

C. G. Mead

The oligodeoxyribonucleotide transferase system is capable of condensing oligodeoxyribonucleotides to form a polydeoxyribonucleotide.[1] The system has been partially purified by diethylaminoethylcellulose (DEAE-cellulose) ion exchange chromatography and ammonium sulfate fractionation. Gel filtration on Sephadex G-100 results in the fractionation of the system into two components, both of which are necessary for activity. These two fractions are being further purified by cellulose ion exchange chromatography so that the individual activities can be characterized. Recombination-deficient mutants of *E. coli* have been obtained from A. J. Clark and are being examined for their complements of oligodeoxyribonucleotide transferase in both the uninfected and T2-infected conditions.

References

[1]C. G. Mead, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1964*, ORNL-3700, p. 151.

17.3 BIOSYNTHESIS OF RNA BACTERIOPHAGE R17: EFFECT OF 5-FLUOROURACIL INCORPORATION

Robert Fujimura

Bacteriophage R17 is a small, spherical nucleoprotein particle containing RNA. The biosynthesis of such a bacteriophage goes through at least three steps. They are formation of a double-stranded RNA intermediate, formation of progeny RNA, and formation of progeny bacteriophage. Thus the system may need at least three kinds of new protein: an enzyme for double-stranded RNA formation, an enzyme for progeny RNA formation, and phage coat protein.

The present study is on the effect of incorporation of 5-fluorouracil (FU) on the expression of the function of viral RNA, that is, the formation of the three kinds of molecules mentioned above. The specific questions under investigation are: (1) When normal bacteriophage is used to infect a cell in the presence of FU at a concentration where maximum inhibition of viable phage occurs, is the expression of all three molecules inhibited? (2) If FU does markedly inhibit the expression of all three kinds, then is it possible to suppress expression of one or the other by adding this analog at different times after infection? (3) If FU does not inhibit the formation of phagelike particles, does the infection of bacteria with such progeny particles initiate any infectious processes?

The results so far show that the syntheses of all three kinds of molecules are markedly inhibited when 0.5 micromole/10 ml of FU is added simultaneously with infection, but some FU-labeled molecules of all three kinds are still formed. The formation of the three species cannot be suppressed much further by addition of higher concentrations of FU or addition of another analog, 8-azaguanine. According to the best available data, viability of phage is decreased to about 0.3% of normal, and the remaining viability is due to newly synthesized phage. Investigation of questions (2) and (3) is in progress.

17.4 A UNIQUE AMINOACYL-sRNA SYNTHETASE

W. E. Barnett

Partial purification of the *Neurospora crassa* enzyme(s) involved in the aminoacylation (charging) of *E. coli* sRNA's with phenylalanine has revealed the presence of an exceptional phenylalanyl-sRNA synthetase. This *Neurospora* enzyme aminoacylates the $sRNA^{phe}$ of *E. coli*, but not the $sRNA^{phe}$ of *Neurospora*, with phenylalanine and may be separated by DEAE-Cl column chromatography from the normal phenylalanyl-sRNA

synthetase of *Neurospora*. This observation is being investigated in detail.

17.5 CODING PROPERTIES OF ANOMALOUSLY CHARGED sRNA'S

J. L. Epler W. E. Barnett

Barnett and Jacobson[1] have reported that preparations of aminoacyl-RNA synthetases from *Neurospora crassa* are capable of attaching L-phenylalanine to the phenylalanyl-sRNA of *E. coli* and to at least two other species of sRNA. The latter are not charged with phenylalanine by the enzymes from *E. coli*, and evidence was presented that one of the sRNA's involved was *E. coli* alanyl-sRNA. In the present study, uncharged *E. coli* sRNA's were partitioned into different phases by the method of Khym, and the alanyl-sRNA and the phenylalanyl-sRNA were separated. The *E. coli* alanyl-sRNA was charged with ^{14}C-L-phenylalanine by the *Neurospora* enzymes and compared with ^{14}C-alanyl- and ^{14}C-phenylalanyl-sRNA's formed by the *E. coli* enzymes. In a polyribonucleotide-dependent aminoacyl-sRNA binding system, the alanyl-sRNA heterologously charged with phenylalanine was found to behave as *E. coli* alanyl-sRNA, not as phenylalanyl-sRNA. These results demonstrate that the synthetases of *Neurospora* are indeed aminoacylating the alanyl-sRNA of *E. coli* with phenylalanine and that the coding properties of the alanyl-sRNA are not influenced by the amino acid to which it is linked.

References

[1]W. E. Barnett and K. B. Jacobson, *Proc. Natl. Acad. Sci. U. S.* **51**, 642 (1964).

17.6 AMINOACYL-sRNA BINDING PROPERTIES OF BIOSYNTHETIC POLYNUCLEOTIDES

J. L. Epler

Polyribonucleotides were synthesized with RNA polymerase from double-stranded deoxy-polymers

Table 17.6.1. Binding of ^{14}C-Aminoacyl-sRNA to Ribosomes Mediated by Deoxypolynucleotide-Directed Ribopolynucleotides

dA : dT + UTP ⟶ poly U
dA : dT + ATP ⟶ poly A
dI : dC + CTP ⟶ poly C
dI : dC + GTP ⟶ poly G

M. lysodeikticus RNA polymerase

Polynucleotide	Micromicromoles of ^{14}C-Aminoacyl-sRNA Bound							
	Lys		Phe		Prol		Val	
	OR[a]	Comm[b]	OR[a]	Comm[b]	OR[a]	Comm[b]	OR[a]	Comm[b]
None	0.8	0.8	0.9	1.3	0.3	0.4	0.8	0.9
A	11.9	3.2	0.5	1.3	0.2	0.4	0.4	0.8
C	0.4	0.7	0.4	1.6	1.0	1.6	0.5	0.7
G	0.8		1.1		0.4		0.6	
GU		0.6		1.8		0.5		6.2
U	0.4	0.7	1.7	11.5	0.3	0.4	0.5	0.8
dA	1.0		0.7		0.3		0.5	
dC	0.7		0.9		0.5		0.6	

[a]OR = Oak Ridge.
[b]Comm = commercial, Miles Chemical Co.

by Bollum.[1] The activity and the specificity in the binding of *E. coli* ^{14}C-aminoacyl-sRNA's to *Neurospora* ribosomes were investigated. Poly A, poly C, and poly U were found to bind ^{14}C-lysyl-, ^{14}C-prolyl-, and ^{14}C-phenylalanyl-sRNA's respectively. The single-chain polydeoxynucleotides, dA and dC, showed little activity.

References

[1]F. J. Bollum *et al.*, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1964*, ORNL-3700, p. 148.

17.7 SEPARATION OF TRANSFER RIBONUCLEIC ACIDS BY A SIMPLE EXTRACTION PROCESS

J. X. Khym

Introduction. – Extension of our liquid-liquid extraction technique,[1] in which amine salts dissolved in selected organic solvents are used to extract nucleic acids, led to the observation that an aliphatic quaternary ammonium chloride dissolved in trichlorotrifluoroethane is markedly selective in extracting various amino-acid-specific transfer ribonucleic acids (tRNA's) from dilute aqueous solutions.

Experimental. – The extractions were carried out with 5.2% quaternary ammonium chloride (tricaprylylmethyl ammonium chloride) dissolved in trichlorotrifluoroethane (Freon TF). Transfer RNA was completely extracted into the Freon layer from sodium chloride solutions less than 0.25 *M* after 5 min of mixing. Several hours of shaking were necessary to establish equilibrium between phases when the aqueous NaCl concentration was between 0.25 and 0.40 *M*. The tRNA in the organic layer was recovered quantitatively by shaking with an equal volume of 0.5 *M* NaCl for 1 hr. All fractions were extensively dialyzed prior to assay for aminoacyl-tRNA formation.

Table 17.7.1. Distribution of Transfer Ribonucleic Acids Between a Quarternary Ammonium Chloride–Freon Phase and 0.32 *M* Sodium Chloride Solution

E. coli tRNA, initially at 3.0 mg/ml in the organic phase, was shaken with an equal volume of 0.32 *M* sodium chloride for 6 hr. Another aliquot of tRNA from the initial organic phase, extracted into 0.5 *M* NaCl, served as the control. The tRNA from both phases and the control was assayed for aminoacyl-tRNA formation, using the ^{14}C-amino acids shown.

^{14}C-Amino Acid Tested	Micromicromoles of Amino Acid Incorporated per Milliliter of Phase			E_a^o Organic/Aqueous	Percent Recovery
	Control	Organic	Aqueous		
Lysine	1080	46	990	0.05	96
Valine	1050	156	820	0.19	93
Alanine	700	110	480	0.24	87
Arginine	1000	235	800	0.29	103
Histidine	660	175	460	0.38	97
Threonine	930	370	620	0.59	106
Leucine	1830	1170	650	1.8	99
Serine	550	390	150	2.6	99
Proline	810	670	135	4.9	99
Tyrosine	420	330	11	30	83
Phenylalanine	740	740	12	62	103

Results and Discussion. – A typical fractionation of the individual tRNA species in such an extraction procedure is illustrated by the following experiment. Transfer RNA, initially at 3 mg/ml in a Freon-amine solution, was shaken for 6 hr with an equal volume of 0.32 *M* sodium chloride solution. Following dialysis, the tRNA from both phases was tested singly with 11 different amino acids for amino acid acceptor activity. The results, given in Table 17.7.1, yield extraction coefficients ($E_a^{\circ} = C_{org}^{\text{acyl-tRNA}}/C_{aq}^{\text{acyl-tRNA}}$, where C = concentration) that differ strikingly.

In a similar experiment, the phases resulting from such a 0.32 *M* sodium chloride extraction were further treated. The aqueous phase was extracted twice with fresh organic solvent, and the organic phase twice more with 0.34 *M* NaCl. The tRNA in both phases was tested for acceptor activity with phenylalanine and alanine. The organic phase contained 48% of the starting phenylalanyl-tRNA and 0.1% of the alanyl-tRNA; the aqueous phase contained 50% of the alanyl-tRNA and 0.3% of the phenylalanyl-tRNA. These findings are consistent with the data given in Table 17.7.1 for these two transfer nucleic acids, and demonstrate that tRNA can be separated conveniently into definite groups by single-batch extractions with this solvent system.

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17.8 NUCLEOTIDE AND NUCLEIC ACID METABOLISM IN NORMAL AND HYBRID AMPHIBIAN EMBRYOS

F. J. Finamore A. H. Warner
R. P. Feldman

Introduction. – In a continuing study of the nucleic acids of amphibian eggs, we have recently undertaken a comparison of normal embryogenesis with lethal hybrid development in an attempt to ascertain the nature of the hybrid block and perhaps thereby obtain evidence as to the role of oligonucleotides and polynucleotides in development.

Methods. – Each of 15 female *Rana pipiens* received an intraperitoneal injection of 250 μc of ^{32}P as orthophosphate and 250 μc of uridine-^{3}H for four consecutive days. On the fifth day each was administered whole frog pituitary glands to stimulate ovulation, and, on the following day, labeled eggs were obtained. The unfertilized eggs from each female were randomly distributed among several dishes; half the eggs were fertilized with homologous spermatozoa, and half were fertilized with spermatozoa from *Rana catesbiena*. At stages corresponding in normal embryos to cleavage, gastrulation, neurulation, and hatching, both normal and hybrid embryos were denuded chemically, and the following extraction procedures were employed:

1. RNA was prepared by the procedure of Kay and Dounce.[1]
2. Acid-soluble fraction, containing free ribonucleotides, was obtained from the residue.
3. After removal of free nucleotides, the acid-insoluble precipitate was extracted with hot NaCl to obtain DNA and residual RNA.

In addition to total ^{32}P and ^{3}H counts and quantitative estimation of concentrations and specific activities of each gross fraction, the following analytical methods were used:

1. The RNA, prepared by the procedure of Kay and Dounce, was subjected to sucrose gradient centrifugation and chromatography on methylated serum albumin columns.
2. Hot NaCl extracts, containing RNA and DNA, were subjected first to ECTEOLA-cellulose column chromatography to separate RNA from DNA, then the RNA was fractionated into oligonucleotides and polynucleotides by DEAE-cellulose column chromatography.
3. DNA, after recovery from ECTEOLA cellulose, was analyzed for radioactivity.
4. Acid-soluble nucleotides were separated by anion exchange chromatography, and concentrations, total ^{32}P and ^{3}H counts, and specific activities were determined for each compound.

Results. *RNA Analysis.* – In normal developing embryos, as we reported previously, there is a measurable *net* synthesis of RNA beginning at gastrulation and continuing through the hatching stage. Of considerable interest, however, is the

fact that hybrid embryos, which undergo developmental arrest at gastrulation, also show net synthesis of RNA even though development has ceased. However, the rate of RNA synthesis in hybrid embryos is approximately half that occurring in normal embryos.

Sucrose gradient centrifugation and methylated serum albumin column chromatography of the RNA revealed that, from gastrulation to hatching, normal embryos synthesize primarily oligonucleotides and 4S RNA. After the normal animals have hatched, ribosomal RNA as well as 4S RNA are synthesized. Hybrid embryos under developmental arrest reveal patterns of RNA synthesis *exactly* like those of normal embryos. Thus, even though development is arrested at gastrulation in hybrid embryos, the sequence and timing of RNA synthesis are apparently the same as in normal developing embryos.

DNA Analysis. – In normal embryos, net synthesis of DNA begins some time during gastrulation and continues through hatching. In hybrid embryos, however, we observed a stimulation of DNA synthesis *before* gastrulation, and net synthesis is maintained at a higher level than normally through stages corresponding to gastrulation and neurulation. After this time, however, net synthesis of DNA in hybrid embryos falls below that occurring in normal embryos.

Acid-Soluble Nucleotide Analysis. – The stimulation of DNA synthesis at early stages of hybrid embryo development is probably reflected by changes occurring in the acid-soluble nucleotide pool. At cleavage, both the concentration and specific activities of the acid-soluble nucleotides are higher in hybrid embryos than in normal embryos. As development proceeds, however, the nucleotide pool in hybrid embryos decreases at a faster rate than normal, and, at a stage corresponding to hatching in normal embryos, the pool of nucleotides is less than half the normal pool.

Discussion. – It is quite apparent from these studies that hybrid developmental arrest is not due to failure of the nucleic-acid-synthesizing machinery. Synthesis of ribosomal RNA, transfer RNA, and DNA, as well as oligonucleotide utilization, continues long after developmental arrest. Perhaps development is blocked because, under the influence of foreign DNA, messages are synthesized that are completely meaningless to the egg-protein-synthesizing machinery, or for some reason there is a failure in delivering the messages to the proper sites in the egg. A further study directed toward messenger RNA synthesis in normal and hybrid embryos therefore seems highly desirable.

Summary. – Lethal hybrid embryos, after developmental arrest, continue to synthesize transfer RNA and ribosomal RNA and to utilize oligonucleotides in a pattern similar to that occurring in normal embryos. However, at the early stages of development of hybrid embryos, there is a stimulation of DNA synthesis that occurs before net synthesis of DNA is observed in normal embryos. The accelerated rate of DNA synthesis continues in hybrid embryos for at least 12 hr after developmental arrest, but, thereafter, synthesis of DNA in normal embryos exceeds that occurring in hybrid embryos.

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17.9 STUDIES ON RNA POLYMERASE FROM AMPHIBIAN LIVER

S. Iyeiri F. J. Finamore

The presence in amphibian eggs and embryos of large quantities of oligoribonucleotides and the marked changes that occur in these compounds with development[1] suggest that the oligonucleotides may function either as precursors of polynucleotides or as regulators of differentiation. We have initiated studies to explore the possibility that oligonucleotides are regulators of differentiation by affecting an RNA polymerase system. This approach necessitates first the isolation, purification, and study of the properties of an RNA polymerase from amphibian tissue.

Methods and Results. – An active, partially purified RNA polymerase was obtained by using a nuclear fraction from liver of *Rana pipiens.* Purification of the enzyme was achieved by nuclear lysis, ammonium sulfate and streptomycin sulfate fractionations, and by ECTEOLA- and DEAE-cellulose column chromatography.

The reaction mixture and assay procedures were as follows: The complete reaction mixture contained 50 micromoles of Tris-HCl buffer, pH 7.4, 30 micromoles of KCl, 5 micromoles of NaF, 8 micromoles of $MgCl_2$, 0.03 micromole of unlabeled ribonucleoside triphosphates, about six optical-density units of native liver DNA from *R. pipiens*, 0.6–0.8 micromole of ATP-^{3}H or UTP-^{3}H, and enzyme, in a final volume of 0.7 ml. At 0, 15, and 30 min, duplicate 100-μl samples were removed and applied to filter paper disks which were subsequently washed in 5% TCA–0.01% ATP, 5% TCA–0.01 *M* PP_i, ethanol-ether, and ether, and then counted.

No incorporation of ATP-^{3}H or UTP-^{3}H into acid-insoluble products occurs in the absence of divalent metal ion, liver DNA, or sodium fluoride. Divalent magnesium is more effective than Mn^{2+} in this system. Incorporation is maximal at about pH 7.0 to 7.5 and is linear with time for 30 min.

Preliminary evidence indicates that amphibian egg oligoribonucleotides are potent inhibitors of liver RNA polymerase.

Experiments are now in progress to determine if an RNA polymerase system, similar to amphibian liver RNA polymerase, is active in frog embryos during embryonic development.

Summary. – A partially purified preparation of RNA polymerase has been isolated from liver nuclei of *Rana pipiens*. The enzyme catalyzes the incorporation of labeled ribonucleoside triphosphates into acid-insoluble products in the presence of ribonucleoside triphosphates, native amphibian liver DNA, sodium fluoride, and a divalent metal ion.

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17.10 OCCURRENCE OF P^1,P^3-DIGUANOSINE 5′-TRIPHOSPHATE IN BRINE SHRIMP EGGS

A. H. Warner F. J. Finamore

Introduction. – The recent observation that P^1,P^4-diguanosine 5′-tetraphosphate (d-GDP) occurs in large amounts in the brine shrimp, *Artemia salina*,[1] prompted an investigation of the acid-soluble fraction of encysted brine shrimp eggs as a preliminary study of the biochemical function of di-GDP during development. These studies indicate the presence of a guanine-containing compound in *Artemia*, similar in structure to di-GDP but differing in its chemical composition. Whereas snake venom phosphodiesterase liberates GMP and GTP from di-GDP, it liberates GMP and GDP from the new compound.

Results. – The compound can be extracted from the encysted brine shrimp egg with 1 *N* $HClO_4$ and readily purified by ion exchange chromatography, using formate and formic acid.[2] Chemical analysis shows the guanine : ribose : total nitrogen : total phosphorus : labile phosphorus content to be 1.00 : 0.93 : 4.8 : 1.52 : 0.45, while the ultraviolet-light-absorbing characteristics of the compound are virtually identical to those for other guanine-containing nucleotides. Although the compound is resistant to attack by alkaline phosphatase, snake venom phosphodiesterase hydrolyzes it completely to 5′-GMP and P_i; however, the initial products formed are 5′-GMP and 5′-GDP in equimolar quantities.

In view of the chemical composition and products obtained on enzymic hydrolysis, we conclude that the compound is P^1,P^3-diguanosine 5′-triphosphate, a homolog of di-GDP, differing from di-GDP by only one phosphate group.

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18. Enzymology

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Protein Synthesis (18.1–18.12)

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Hormonal Control (18.13–18.16)

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Mammalian Cell Regulation (18.17)

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Cooperative NIH-AEC Co-Carcinogenesis Program (18.13–18.15, 18.16)

18.1 STUDIES ON THE CELL-FREE SYNTHESIS OF β-GALACTOSIDASE

Lawrence Rosen G. D. Novelli

Introduction. – Attempts to obtain a stable system which synthesizes β-galactosidase in vitro have continued. Such a system requires a high-speed supernatant factor (S_{90}) and a particle fraction.[1,2] Recently, two approaches have been made to obtain a useful particle fraction.

Results and Discussion. – The first approach to isolate a particle fraction from *E. coli* was by the method of Nisman *et al.*[3] An inducible strain of *E. coli* is grown in the absence of inducer, then converted to spheroplasts by the use of penicillin. The spheroplasts are harvested, washed, and subsequently lysed with digitonin. Differential centrifugation leads to a complex particle fraction, P_1, which, in the presence of induced and added cofactors, synthesizes β-galactosidase. Although P_1 did not require an S_{90} fraction for β-galactosidase synthesis, the addition of S_{90} did increase the amount of enzyme synthesized. However, P_1 rapidly loses its synthetic ability and apparently cannot be stored. For this reason, experiments with this material were discontinued.

The second approach has been to treat *E. coli* cells with a mixed-bed resin prior to passage through the French pressure cell at low pH (4 to 5).[4] After passage through the pressure cell, the cells are lysed by raising the pH to the range 6 to 7. The lysed exudates are then centrifuged to obtain an S_{90} fraction and two particle fractions, a 30,000 × *g* pellet (P_{30}), and a ribosomal fraction (P_{90}).

Generally, the P_{30} fraction has been found to be active in the synthesis of β-galactosidase in vitro when supplemented with S_{90} and other factors. The P_{90} fraction, under identical conditions of incubation, has been found to be less active.

The major parameter which appears to affect the amount of β-galactosidase synthesis is the pH to which the French cell exudate is brought. Since the degree of lysis which occurs is pH and time controlled, a major consideration at present is to obtain better control of lysis. Such lysis should enable us to maximize the yield of the particle fraction necessary for the in vitro synthesis of β-galactosidase.

Experiments are now in progress to isolate the particle fraction by the use of sucrose density gradients.

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18.2 AMINO ACID INCORPORATION BY EXTRACTS OF *ESCHERICHIA COLI*

J. V. Schnell G. D. Novelli

Introduction. – As previously reported,[1] it is possible to damage bacteria without releasing cytoplasmic components by passage of the bacterial suspension through the French pressure cell at low pH. Lysis will occur when the suspension of damaged bacteria is adjusted to neutral pH. Studies on this phenomenon and on the amino-acid-incorporating activity of the subcellular extracts are being continued.

Results and Discussion. – By use of turbidimetric and spectrophotometric measurements it is possible to study the lysis of the damaged bacteria. When a suspension of damaged bacteria is neutralized, the turbidity immediately decreases by about 70%. That this represents release of cytoplasm is indicated by a tenfold increase of material absorbing light at 260 and at 280 mμ after whole cells and debris are removed by low-speed centrifugation. The lysis is apparently a general effect of pH, since the nature of the base used to neutralize the suspension does not affect the extent of lysis. Although ionic strength may also affect the lytic process, results suggest that pH is primarily the controlling factor.

In all but one case the extracts prepared by the lysis of damaged bacteria have been more active for the incorporation of labeled L-leucine than have extracts prepared from bacteria disrupted by the French pressure cell in the conventional manner. Sucrose-density-gradient centrifugation did not indicate increased polysome populations in the lysates of damaged bacteria. At present no explanation for the enhanced activity is evident.

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18.3 ISOLATION AND PURIFICATION OF AMINO-ACID-ACTIVATING ENZYMES

M. P. Stulberg

Introduction. – Our efforts to purify the isoleucine-activating enzyme (IAE) from *E. coli* have resulted in the production of a highly purified fraction. Concurrently, we are isolating the phenylalanine-activating enzyme (PheAE) from the same source.

Results and Discussion. – A purification protocol consisting of cell breakage, centrifugation to remove particulate matter, protamine precipitation for removal of nucleic acids, ammonium sulfate fractionation, and DEAE and hydroxylapatite column chromatography, have produced an approximately 2000-fold purified IAE. At the present time, the amounts isolated are too small to quantitatively determine absolute purification on the basis of physical studies. Interfering substances prevent an exact determination of the amount of protein present. Future work will consist in preparing larger amounts of the enzyme so that an accurate characterization of the enzyme can be concluded.

The purification of the PheAE was initiated from the eluate of the DEAE chromatography. All activity was located in the same fractions that contained the IAE activity. Hydroxylapatite chromatography separated the two activities completely. The PheAE appears quite homogeneous and is free from all other amino-acid-activating activities except for tyrosine. The peak of the tyrosine-activating enzyme is slightly removed from that of the phenylalanine enzyme under the present conditions of hydroxylapatite chromatography. Other methods will be utilized to obtain further separation.

The PheAE is currently the object of most of our efforts because of the significant purification of phenylalanine tRNA (Phe tRNA).[1] When the enzyme is obtained free of other activities and in a more homogeneous state, we will initiate studies on the interaction of the enzyme and the tRNA. Our goal is to obtain information on the enzyme recognition site for the tRNA molecule. This recognition seems to be absolutely specific in contrast to results in experiments with the genetic code where much degeneracy exists.

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18.4 ISOLATION AND PURIFICATION OF SPECIFIC TRANSFER RNA FROM *E. COLI*

A. D. Kelmers[1] G. D. Novelli
M. P. Stulberg

Introduction. – Since our last report,[2] we have improved methods for the chromatographic separation of the tRNA's. The separation of phenylalanine tRNA (Phe tRNA) from the other tRNA's has progressed significantly.

Results and Discussion. – The utilization of 8-ft columns with an elution period of three days has accomplished significant resolution of the tRNA's. Amino acid acceptor assays have demonstrated partial resolution of 16 tRNA's. Multiple peaks have been observed for many of the amino acids; leucine activity has been accounted for in five separate fractions. Phe tRNA emerges last from the column with only a slight contamination from leucine and tyrosine tRNA's. Rechromatography of the Phe tRNA peak on a 2-ft column has produced a fraction that assays as 63% pure Phe tRNA and 84% pure tRNA. We believe these to be minimum values of purity, limited by our present information on counting efficiency and molar extinction coefficients.

Presently we are attempting to eliminate the contaminations of Phe tRNA by leucine and tyrosine tRNA's. Other partially separated peaks of activity will be further resolved for individual tRNA isolation.

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18.5 POLYRIBOSOME FORMATION AND AMINO ACID INCORPORATION DURING LIVER REGENERATION

Piero Cammarano R. D. MacElroy
G. D. Novelli

Introduction. – This is a continuation of our studies of the factors that control protein synthesis during liver regeneration.[1]

Results and Discussion. – Amino acid incorporation by microsomal systems from regenerating liver increased linearly with time after surgery, which is attributable to an increase in the intrinsic activity of the microsomes during liver regeneration. Supernatants from normal livers suppressed the activity of both microsomes and ribosomes from regenerating liver, indicating the presence of some inhibitory factors in supernatants from normal liver.

Sucrose-density-gradient analysis of postmitochondrial supernatants revealed that during liver regeneration there is a striking increase in heavy polysomes at the expense of lighter aggregates and monosomes. Since in vivo studies with ^{14}C-leucine showed that the monomers are inactive in protein synthesis, the increase in heavy polysomes during liver regeneration can account for the increase in protein synthesis.

Centrifugation of the postmitochondrial supernatants through a discontinuous gradient with and without deoxycholate shows the majority of polyribosomes to be tightly bound to membranes in normal liver but either free or loosely bound in regenerating liver. The significance of this loose association of the polyribosomes and membranes during liver regeneration is not readily apparent, but, since the bulk of protein synthesis is carried out by polyribosomes attached to membranes, this observation suggests that during liver regeneration there is a reorganization at the membrane level that may permit a more ready attachment between ribosomes and messenger RNA.

Studies with polyuridylic acid directing the incorporation of ^{14}C-phenylalanine have indicated an anomalous behavior of the in vitro incorporating systems. Microsomes from regenerating liver are more responsive to poly U than normal microsomes are. This difference disappears at the ribosomal level. Sucrose-density-gradient analysis of reaction mixtures shows that poly U only interacts with monomers. The anomalous finding is that, although there is a decrease in the size of the monomer pool during liver regeneration, the microsomes are more responsive to poly U than are normal microsomes. Thus there may be factors at the membrane level that permit a more ready association of monosomes with poly U. An alternative explanation would be that the monomer pool consists of a mixture of active and inactive monosomes, that is, active monomers would be capable of interaction with poly U, while the inactive ones would not. Thus, a change in the ratio of active to inactive monomers during liver regeneration would account for the above finding.

The striking difference between supernatants derived from normal and regenerating liver is again evident in the poly U experiments. It is difficult to decide whether the greater activity of supernatants from regenerating liver is a consequence of the loss of inhibitory factors or an increase in stimulating factors.

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18.6 PROBLEM OF THE DETERMINATION OF THE HALF-LIFE OF MESSENGER RNA IN RAT LIVER BY MEANS OF ACTINOMYCIN D

Marialuisa Melli F. M. Ritossa
G. D. Novelli

Introduction. – Many studies with bacteria have yielded information leading to the conclusion that messenger RNA (mRNA) has a short half-life of the order of 2–3 min. The prolonged synthesis of hemoglobin in rabbit reticulocytes in the absence of continued RNA synthesis has raised the question of whether mRNA in mammalian cells is relatively stable in contrast to the situation with bacteria. Since actinomycin D is known to inhibit most RNA synthesis, this compound has been used in attempts to measure the half-life of mRNA in bacteria and in mammals.[1,2] Staehelin *et al.*[3] have shown that the injection of actinomycin into rats results in the breakdown of rat liver polysomes and concluded that this is a consequence of the breakdown of mRNA. Hiatt,[2] using doses of actinomycin that inhibit nuclear RNA synthesis only 47% or less, concluded that the half-life of rat liver mRNA is of the order of 40 hr. However, Trakatellis *et al.*,[4] working with mouse liver, have proposed that, after actinomycin treatment, mRNA stored in the nucleus enters the cytoplasm, causing a longer apparent half-life of mRNA.

Since there is no really adequate definition of mRNA, we decided to measure the loss of ability of rat liver to synthesize protein as a function of time after injection of actinomycin and thereby to obtain a measure of the half-life of the protein-synthesizing unit which should include mRNA.

Results and Discussion. – Rats were injected with doses of actinomycin sufficient to completely inhibit RNA synthesis within 10 min after injection. In vivo protein synthesis was measured 2, 3, 6, and 9 hr after actinomycin treatment by giving the animals a 20-min pulse of ^{14}C-leucine. The specific activity of the liver protein was measured as usual.

In parallel experiments, microsomes were obtained at 2, 3, 6, and 9 hr after actinomycin treatment, and protein synthesis was measured in vitro.

The in vivo protein synthesis decayed exponentially with a half-life of about 5 hr. The in vitro amino acid incorporation by microsomes decreased to a value of 37% of the control level and then leveled off. The half-life of the decrease was about $2\frac{1}{2}$ hr. Since the microsomes were tested with supernatants from the same animal, we repeated the incorporation studies with the microsomes, using a supernatant from a normal animal, and obtained the same results. This experiment demonstrated that the effect of actinomycin is clearly at the microsomal level. We, therefore, prepared ribosomes from the livers of treated animals and determined the distribution of the various-sized polysomes by sucrose-density-gradient analysis. We observed that treatment with actinomycin results in a large decrease of the very heavy ergosomes, with a corresponding rise in the monomer peak. Thus the loss of protein-synthesizing capacity can be accounted for by this large loss of ergosomes.

The discrepancy between our data and other published data and between our own in vivo and in vitro results is difficult to explain. However, the full consequences of actinomycin treatment are not known. Actinomycin may have toxic properties unrelated to its inhibition of RNA synthesis, and the effect on protein synthesis may be indirect through some other mechanism. Until we have some independent and specific measure of mRNA, these results must remain equivocal.

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18.7 PROTEIN SYNTHESIS BY PREPARATIONS FROM LIVERS OF THE RAINBOW TROUT

E. L. Murray G. D. Novelli

Introduction. – This represents the initiation of a new program in the chemical carcinogenesis program. It appears that some strains of rainbow trout are particularly sensitive to chemical carcinogenesis, especially of the liver.[1] Some fish populations appear to be 100% affected.[2] Although spotted observations of trout hepatoma appear in the earlier literature, it was not until about 1960 that the massiveness of this condition in hatchery rainbow-trout populations became evident.[2] The etiology of the condition is not yet established, but it appears to be associated with the use of dry feed, pelleted diets which have been used for fish food for a relatively short time in this country.

The importance to cancer research of the finding of an animal population that exhibits 100% hepatomas is obvious.

We have, therefore, undertaken an investigation that will attempt to understand what biochemical changes take place in the trout liver leading to the development of hepatoma.

A review of the literature revealed that nothing is known concerning the metabolism of trout liver or, as a matter of fact, of fish liver in general. Therefore, we need to develop some basic parameters regarding the metabolism of normal trout liver before undertaking studies with trout hepatomas. Because we feel that a liver undergoing the development of a hepatoma will be most likely to exhibit changes in macromolecular synthesis, our initial efforts have been to study the in vitro incorporation of amino acids by subcellular preparations from trout liver.

Results and Discussion. – Using techniques we have developed for studies with rat liver, we examined the C-ribosome profile in trout liver by sucrose-density-gradient analysis. These profiles were similar to those in rat liver except that the monosome peaks were much larger and the polysome regions gave less resolution of peaks.

We used these C ribosomes to develop a system for the incorporation of amino acids into protein. This system has about the same efficiency for incorporation as does the similar system from rat liver, and has similar energy and metal requirements. However, when poly U was used to stimulate phenylalanine incorporation, the trout system was much less efficient than the rat liver system. It is possible that we have not yet found optimal conditions for the poly U system.

When we studied the effect of temperature on the incorporation by the trout liver system, we found that 23° was optimal for this system in contrast to 37° for the rat liver system. Crossing fractions between trout liver and rat liver showed that the optimal temperature was determined by the source of the supernatant. Thus a system consisting of a supernatant from trout liver with rat liver ribosomes had an optimal temperature of 23°, whereas the reciprocal combination had an optimal temperature of 37°.

A careful study of the partial reactions, that is, the charging of transfer RNA and the transfer of the amino acid from aminoacyl-sRNA to ribosomes showed that the transfer enzymes in trout liver are temperature sensitive.

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18.8 STUDIES ON THE INCORPORATION OF ^{14}C-UTP INTO RNA OF ISOLATED TROUT LIVER NUCLEI

D. P. Kosow G. D. Novelli

Nuclei have been isolated from trout liver by homogenization in a Teflon-glass homogenizer in isotonic sucrose followed by low-speed centrifugation. The nuclear pellet is then resuspended in nine volumes of 2.2 *M* sucrose and centrifuged at 80,000 × *g* for 100 min. Under these conditions, erythrocytes and cytoplasmic contaminants float and the nuclei sediment. The nuclei prepared by this procedure are virtually free of contaminants as judged by phase-contrast microscopy. These purified nuclei are being used to study the in vitro incorporation of ^{14}C-UTP into RNA. The incorporation is proportional to the amount of nuclei added and is 80% complete after 10-min incubation. The optimal conditions for the reaction are incubation at 30° in the presence of (in micromoles per milliliter): potassium phosphate buffer, pH 7.5, 20; ATP, 2; CTP, 1; GTP, 1; phosphocreatine, 10; $MnCl_2$, 2; 20 μg creatine kinase; and 0.54 μc of ^{14}C-UTP. Dextran 500 (2.5%) is added to prevent gelling of the nuclei.

The product of the reaction has been isolated by phenol extraction at 60° in the presence of sodium lauryl sulfate. When analyzed by sucrose gradient centrifugation, the product sediments as one broad band between 4S and 40S, with a peak at about 28S.

18.9 STUDIES ON THE EFFECT 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 seedlings have been studied further. Albino maize mutants have been used in an effort to demonstrate a relationship between the light response under investigation and the light-induced initiation of chloroplast development. Alternate methods of particulate preparation and the effects of polyuridylic acid (poly U) on the activity of these preparations also have been examined.

Results and Discussion. – Normal and albino mutant maize seedlings of the W_1 and W_3 strains were exposed to light under conditions described previously. Ribosomes prepared from normal light-treated seedlings of either strain exhibit an enhanced capacity for amino acid incorporation when compared with control ribosomes from similar seedlings maintained in the dark. The magnitude

of this stimulation (60 to 70%) is similar to that reported earlier[1] for other strains of maize. However, the activity of ribosomes from mutant W_1 seedlings is unaffected by the light treatment, and ribosomes from light-treated mutant W_3 seedlings are stimulated less than 20%. Light-induced chloroplast development in both mutant strains is reportedly arrested; thus these results provide additional support for the earlier observation that factors affecting chloroplast development similarly affect the in vitro ribosomal system under study.

Attempts to prepare microsomes from etiolated maize shoots and bean leaves by conventional techniques normally employed with rat liver have failed to yield a particle fraction which differs significantly from ribosomes in RNA and protein content or in amino-acid-incorporating activity. The activity of "microsomal" preparations is stimulated to the same degree following light treatment as that observed normally with ribosomes.

Incorporation of ^{14}C-phenylalanine by ribosomes from light-treated and control plants is stimulated eight- to tenfold by the addition of poly U. Under standard assay conditions, the differences in activity between the two ribosomal preparations in the presence of poly U are retained in nearly the same proportion. However, titration of the incorporating systems from treated and control plants with various levels of Mg^{2+} reveals a distinct difference between the two systems in the optimal level of this cation in the presence of poly U. Under different assay conditions (with respect to Mg^{2+}), the addition of poly U abolishes a portion of the stimulation. This difference in Mg^{2+} sensitivity between the two preparations is not observed when leucine incorporation is measured, and is much less marked when phenylalanine incorporation is measured in the absence of poly U.

Further studies will be carried out in an effort to clarify this observed difference between the two preparations.

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18.10 EFFECT OF ACTINOMYCIN D ON THE IMMUNE RESPONSE TO TWO ANTIGENS GIVEN IN SEQUENCE

C. J. Wust M. G. Hanna, Jr.

The injection of actinomycin D into mice 7 hr prior to the injection of sheep erythrocytes (SRBC) results in a 3-day delay in the appearance of circulating hemagglutinin, but it neither affects the rate nor inhibits the amount of antibody produced. Hemagglutinin is demonstrable at 2 days in animals receiving SRBC only, while antibody is not found until 5 days in animals given actinomycin and SRBC. At 2 days with actinomycin treatment, cell destruction in the germinal centers of the white pulp of the spleen is most pronounced, leaving a residual reticular remnant.[1] The large pyronin-staining cell is the principal cell type undergoing pycnosis and phagocytosis. At 6 days, restitution and repopulation by the large cells surviving in the lymphocyte mass have occurred. In experiments designed to study the immune response to a second antigen during the time of maximum cellular injury and the recovery, LAF_1 mice injected with actinomycin before the intravenous injection of SRBC were given rat erythrocytes (RRBC) at 2 or 6 days after the first antigen. In animals that received actinomycin and SRBC, the injection of RRBC at 2 days resulted in the appearance of anti-RRBC within 8 days. This is a delay in the response to the second antigen, since antibody usually appears within 3 days after injection. Antibody to SRBC developed as expected by the fifth day. An interesting observation was made in mice receiving SRBC and RRBC at 2 days. No hemagglutinin to RRBC was detected, and the animals were considered refractory to the second antigen. When RRBC were given at 6 days, hemagglutinin to both antigens appeared, but at a lower level than expected. It was of interest that even at 6 days following actinomycin treatment, when recovery is well established, there was a 1-day delay in the appearance of anti-RRBC hemagglutinin.

A second series of experiments was undertaken to consider the reciprocal sequence of injections, that is, RRBC as the first antigen and SRBC at 2 or 6 days as the second antigen. A delay of 6 to 7 days was observed in the response to RRBC with actinomycin treatment. This delay was evident when SRBC were given at 2 or 6

days. The treatment with antibiotic also caused a delay in the response to SRBC. The interesting feature of the reciprocal sequence, however, is that the animals receiving no antibiotic were not refractory to the SRBC. The injections of the drug and SRBC had no effect on the titers observed to RRBC or SRBC.

The findings may be summarized as follows:

1. In mice that received SRBC followed by RRBC, there was a refractory period to the second antigen.
2. In animals that received actinomycin and SRBC, there was no refractory period to the second antigen.
3. When the reciprocal sequence of antigens was given, that is, RRBC followed by SRBC, there was no refractory period.
4. When the second antigen was given at 2 days to animals that were treated with actinomycin, there was a delay in the appearance of circulating hemagglutinin to it as well as to the first antigen.
5. This delay following actinomycin treatment was also seen when the second antigen was given at 6 days.

In animals that receive actinomycin and antigen, we might speculate that actinomycin not only blocks replication and transformation leading to death and phagocytosis of the large lymphoid cell but also possibly interferes with these cells becoming committed to the antigen. These cells would comprise the few cells seen in the lymphocyte mass that seem to survive the effect of actinomycin. Recovery of these cells from the effect of actinomycin would allow eventual response to either the first or the second antigen.

The difference in response to the second antigen appears to reflect the distinction of closely related and distantly related antigens pointed out by Makinodan and Gengozian (1958).[2]

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18.11 LACTIC ACID DEHYDROGENASE ACTIVITY AS AN INDICATOR OF RAT ERYTHROCYTES DURING THE IMMUNE RESPONSE

C. J. Wust J. B. Murphy

Following electrophoresis on polyacrylamide gel at pH 8.5, isozymes of lactic acid dehydrogenase (LDH) of the mouse migrate entirely toward the anode from the origin. It was found that the LDH of rat erythrocytes (RRBC) moves toward the cathode under the same conditions. Thus, in mixtures of RRBC and homogenates of mouse tissues, the LDH of RRBC could be quantitatively separated and determined. $1C_3F_1$/Cum mice were immunized intravenously with 1 ml of a 10% suspension of RRBC. The plasma, erythrocytes in blood, and homogenates of several organs were subjected to electrophoresis. After completion of electrophoresis, the positions of enzyme were detected by the usual staining technique, and the relative amount of stain was evaluated in a densitometer. About 99.7% of the LDH of RRBC was removed from the circulating pool of erythocytes within the first hour after injection of antigen. The remaining 0.3% stayed at a relatively constant level during the next 2 hr but disappeared from the pool at 3.5 hr. The spleen was the only organ in which the LDH of RRBC was detected, and 60% of the total injected dose was shown to be present by the second hour. At 4 hr, the LDH of RRBC origin in the spleen had decreased to about 20% of the value found at 2 hr. Following this decrease, the amount gradually increased until, at 16 hr after injection, 80% of the injected dose was detected in the spleen. The decrease in the spleen at 4 hr was concomitant with the appearance of soluble rat LDH in the plasma; the LDH reached a maximum at 8 hr and then decreased to 50% in 13 hr. These findings suggest that RRBC were phagocytized primarily in the spleen within 3 hr after injection. The LDH of the RRBC seems to be released from the phagocytes as soluble enzyme into the plasma and finally to be removed by the spleen.

The migration pattern also suggests that the soluble enzyme was not associated with nucleic acid after passage through the spleen, since the association of an oligonucleotide with the enzyme should have been sufficient to cause migration toward the anode. Attempts were made to determine if any LDH band in the gel was associated

with nucleic acid. $1C_3F_1$ mice were immunized with 1 ml of a 10% suspension of rat erythrocytes. Two and one-half hours prior to killing, 10 μc of ^{32}P was injected intraperitoneally at 30-min intervals until 30 μc was administered. Plasma, erythrocytes in the circulation, and homogenates of spleen were prepared and subjected to electrophoresis on polyacrylamide gel. No enzyme band was found with radioactivity.

Experiments are now in progress to determine the fate of the LDH of RRBC in adrenalectomized animals and in animals treated with actinomycin D.

18.12 PHYSICAL STUDIES ON CRYSTALLINE BACTERIAL LUCIFERASE FROM *PHOTOBACTERIUM FISHERI*

Peter Pfuderer Seishi Kuwabara[1]
Daniel Holladay Milton Cormier[1]

Introduction. – *Photobacterium fisheri* is a rod-like luminous marine organism of the order *Pseudomonadeles*. Luciferase, the light-producing enzyme of this organism, will produce visible light if given $FMNH_2$, O_2, and a straight-chain unsaturated aldehyde. The enzyme is closely associated with a DPNH oxidase which reduces FMN. Since the enzyme was recently crystallized for the first time by Cormier and his associates, it was of interest to examine the physical properties of the purified protein.

Results. – Sedimentation studies on luciferase were complicated by the existence of a monomer-polymer equilibrium. The highest polymer of the enzyme had a sedimentation constant of roughly 5S and is presumably identical to the species isolated by Hastings and Riley; it has a molecular weight of approximately 76,000. Evidence was also found for the existence of 4S and 2S species. The 2S species was found to have a molecular weight of 19,000 and to exhibit both DPNH oxidase and luciferase activity. These two activities were found to be slightly separable by chromatography, implying the existence of two different 2S monomers, one with luciferase activity and one with DPNH oxidase activity.

The 4S species contained only luciferase activity, while the 5S species exhibited both enzyme activities. The data for the molecular weight of the 4S species, although inconclusive, since this polymer could not be obtained pure, suggest that it also is composed of four monomers of molecular weight 19,000.

References

[1]Department of Chemistry, University of Georgia.

Hormonal Control

Our analyses of the stimulation of RNA synthesis by steroid hormones are now completed. In two hormone–target organ systems examined, the steroids were found to increase the rate of synthesis of all three of the major RNA species. This result is consistent with a general increase in protein synthesis but not with selective enzyme induction, and we are searching for explanations for the apparent specificity of enzyme induction by hydrocortisone. Our results in these experiments have led to an appreciation of the importance of enzyme degradation in metabolic control, and we have initiated a study of selective enzyme degradation in vitro. The hormonal mode of enzyme induction is also being studied in the Reuber hepatoma, a neoplastic "mutant" of rat liver that is carried in tissue culture. There is a significant difference in the response of these cells that may be of importance in defining neoplastic growth.

18.13 STIMULATION OF TRANSFER RNA SYNTHESIS BY STEROID HORMONES[1]

W. D. Wicks F. T. Kenney

Introduction. – We have previously described[2] a two- to threefold stimulation of RNA synthesis in rat liver and seminal vesicle evoked by hydrocortisone and testosterone respectively. Using a modified thermal phenol extraction procedure, we have shown[2] that both hormones produce an apparently nonspecific increase in the synthesis of high-molecular-weight RNA components as revealed by pulse labeling with ^{32}P. Some question was raised, however, as to the nature of the labeled low-molecular-weight RNA components and possible hormone effects on their synthesis. Recent work has clarified this question.

Results and Discussion. – Tissue, either seminal vesicle or liver, was obtained from castrated or adrenalectomized rats at brief intervals (50 min for seminal vesicle and 20 min for liver) after the injection of ^{32}P (1–3 mc). RNA was isolated and fractionated by the modified thermal phenol procedure.[2] Fraction 1, obtained at 4°, contained all the low-molecular-weight labeled RNA in both organs. Transfer RNA was separated from the other labeled and unlabeled RNA in fraction 1, either by chromatography on DEAE-Cl^- paper or by treatment with 1 *M* NaCl.[2] Separation of transfer RNA was verified by measurements of aminoacyl acceptor activity.

In liver, the effect of hydrocortisone on the labeling of transfer RNA was much lower than that on the high-molecular-weight components. Alkaline hydrolysis, however, revealed that 60–70% of the ^{32}P was present in 2′,3′ CMP, suggesting extensive turnover of the —CCA terminus of transfer RNA. Although the hormone produced only a small increase in the content of ^{32}P in the isolated 2′,3′ CMP (~40%), a two- to threefold increase was observed in the labeling of the other three 2′,3′ nucleotides of hydrolyzed transfer RNA. This indicated that, although the terminal labeling of transfer RNA was relatively unaffected by the hormone, the synthesis of the chain of transfer RNA was markedly enhanced. To test this possibility, transfer RNA, as isolated above, was incubated with purified snake venom phosphodiesterase at 15°C. This treatment has been shown to remove only the three terminal nucleotides of transfer RNA.[3] About 50–70% of the ^{32}P in transfer RNA was rendered acid soluble by enzymatic digestion. More than 95% of this ^{32}P was released as AMP, with the remainder as CMP. The ^{32}P content of the acid-soluble AMP was only slightly increased by hydrocortisone (~30%). In contrast, the phosphodiesterase-resistant RNA showed a two- to threefold increase in labeling, and the isotope was distributed among the four nucleotides in a fashion similar to the base composition of transfer RNA. Thus it is clear that the synthesis of transfer RNA is stimulated by hydrocortisone to the same extent as the high-molecular-weight RNA components, while turnover of the —CCA terminus of transfer RNA is relatively unaffected.

In seminal vesicle there was no indication of turnover of the terminal nucleotides of transfer RNA. Testosterone produced a marked increase in the synthesis of transfer RNA, however, and to an extent that was similar to that of the high-molecular-weight RNA. The isotopic base composition was similar to the chemically determined base composition of transfer RNA. The apparent absence of end labeling of transfer RNA in this organ remains to be explained.

These results are consistent with our earlier work which had suggested that a generalized increase in RNA synthesis resulted from treatment with these two steroids. We now have shown that the synthesis of transfer RNA, ribosomal precursor RNA, and DNA-like RNA in liver is stimulated by hydrocortisone to an extent comparable with that produced by testosterone in seminal vesicle. The basis for the apparent lack of specificity in the response of RNA synthesis to these hormones is currently being investigated.

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18.14 GROWTH HORMONE EFFECTS ON HEPATIC RNA AND ENZYME SYNTHESIS[1]

F. T. Kenney J. Kendrick

Introduction. – Our studies with adrenal steroids have led us to the conclusion that these hormones initiate a general increase in RNA and protein synthesis, and we surmise that enzyme induction is simply a reflection of rapid enzyme turnover. If this is correct, any means by which hepatic protein synthesis is stimulated should result in an apparent induction of the same enzymes that are induced by hydrocortisone. There are several reports of stimulation of hepatic protein synthesis by growth hormone (GH); Talwar, Gupta, and Gros have described[2] GH effects on RNA synthesis that are essentially identical to those we found with hydrocortisone.

Results and Discussion. – A comparison of GH effects in adrenalectomized and intact rats was

made to exclude the possibility of adrenocorticoid involvement. Using commercial sources of GH, we found that GH stimulates RNA synthesis and induces hepatic enzymes only in the presence of functional adrenals, that is, adrenalectomized rats do not respond. We are repeating these experiments using GH preparations[3] of known potency and with known amounts of contaminating ACTH. This should enable us to determine whether the effects of GH are due only to adrenal stimulation, or if the pituitary hormone requires adrenal steroid acting synergistically in order to affect macromolecular syntheses in the liver. In either case, if we are unable to stimulate RNA synthesis independently of adrenal steroid, it is clear that our primary question – whether we can induce hepatic enzymes with agents other than steroid hormones – cannot be answered by these studies. Other agents reported to stimulate hepatic RNA synthesis are being studied in this regard.

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18.15 SELECTIVE DEGRADATION OF "FAST TURNOVER" ENZYMES OF LIVER[1]

W. D. Wicks F. T. Kenney

Introduction. – Adrenal steroids promote an increase in nuclear RNA synthesis, and this is followed by rapid increases in the level of certain hepatic enzymes. The hormone stimulates synthesis of transfer and ribosomal RNA, as well as the DNA-like RNA, so it would seem that selective gene activation is not operative in the response to hormone. The apparent discrepancy between the general nature of the RNA response and the selectivity of enzyme induction can be explained by the inherent differences in turnover rates of the various enzyme proteins. Thus, a general increase in protein synthesis results in large increases in the levels of those enzymes that undergo rapid turnover. This interpretation focuses attention on the importance for metabolic control of the different turnover rates of different enzymes, since it is these differences in turnover rates which determine the rate of response to the hormonal stimulus. Why different enzyme proteins are degraded at different rates and the enzymatic mechanism of this degradation are completely unknown. In these initial studies of enzyme degradation we have defined an experimental system that will enable us to analyze these problems.

Results and Discussion. – When rat liver extracts are incubated at 37°, there is a variable loss of activity of the rapid-turnover enzyme tyrosine transaminase ($t_{1/2} \cong 2.5$ hr, in vivo). Further study of this in vitro degradation has shown that: (1) only the liver soluble fraction (105,000 × *g* supernatant) is required, (2) aging or dialysis of liver soluble fractions increases the rate of degradation, (3) addition of cysteine plus one or two other amino acids markedly increases the degradative rate, but cysteine alone is ineffective, (4) degradation can be completely prevented by supplementation of the reaction mixture with pyridoxal phosphate, the coenzyme for the transaminase, and (5) the functionally related but slow-turnover enzyme glutamic-alamine transaminase ($t_{1/2} \cong 2.5$ days, in vivo) is stable with or without coenzyme addition. We consider the protective effect of coenzyme particularly significant, since pyridoxine can induce the tyrosine transaminase in vivo by preventing enzyme degradation.[2] This observation, together with the correlation of in vitro degradative rates with in vivo turnover times for the two enzymes, lends confidence to the assumption that these in vitro phenomena are a reflection of true enyzme turnover.

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18.16 HORMONAL ENZYME INDUCTION IN THE REUBER HEPATOMA[1]

F. T. Kenney R. L. Tyndall[2] J. Kendrick

Introduction. – The Reuber hepatoma is a slow-growing "minimal deviation" tumor developed from

the livers of rats treated with chemical carcinogens. Techniques for growing this tumor in culture have been worked out by Morse and Potter,[3] who also found that the hepatoma cells respond to hormonal stimulation of enzyme synthesis. We have initiated a study of enzyme induction by hydrocortisone in these cultures, in order to compare the response, in detail, with that in normal liver.

Results and Discussion. – In our view, hydrocortisone treatment of adrenalectomized rats initiates a very general growth response in the liver, with enzyme induction being one consequence of this response. The growth response is temporary, however, for synthesis of the induced enzymes comes to a complete stop a few hours after hormone administration. There is now good evidence that the cessation of enzyme synthesis reflects the action of a cytoplasmic repressor.[4] These observations can be interpreted as indicating that the normal liver has the capacity to limit the growth response initiated by hydrocortisone. When hydrocortisone is added to cultures of the Reuber hepatoma, the tyrosine transaminase level is increased at a rate about half that observed in normal liver. The new level is maintained for as long as 24 hr without any indication of repression. This could be due to a number of factors, such as inability of the cultured cells to metabolize the steroid to inactive forms. It could also reflect inability of the tumor to limit the growth response induced by hydrocortisone. Further study of this phenomenon, with tumors growing in host animals as well as in culture, are planned in order to distinguish these possibilities.

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18.17 ALCOHOL DEHYDROGENASE IN DROSOPHILA: ISOZYMES AND THEIR GENETIC CONTROL

E. H. Grell[1] K. B. Jacobson J. B. Murphy

Introduction. – The genetic control of isozyme forms of several enzymes has been postulated. Evidence for such control has not appeared, except in partial form, for lactate dehydrogenase since phenotypic variants of the two parental types of the enzyme have not been found. In attempting to find such variants in Drosophila, no lactate dehydrogenase of any kind was found, but an alcohol dehydrogenase (ADH) system of isozymes was discovered for which genetic control is demonstrable. Other characteristics of this system will be mentioned also.

Results and Discussion. – Multiple forms of alcohol dehydrogenase may be demonstrated by crushing one specimen of Drosophila on a small strip of filter paper, applying the paper to the end of a polyacrylamide gel, conducting electrophoresis, and employing an assay in which an insoluble reduced tetrazolium is produced as the final electron acceptor and ethanol is the electron donor. Among a hundred or more inbred stocks examined, two electrophoretic patterns of alcohol dehydrogenase isozymes were observed, a fast group and a slow group, and these patterns overlap each other in the gel. Hybrids of two dissimilar strains show all the isozyme bands of the parents plus additional bands in intermediate positions. For example, considering the major band of ADH activity, the hybrid contains a fast band, a slow one, and a band halfway between them. The minor bands behave similarly. Such a three-banded pattern in the hybrid indicates that the ADH of Drosophila may be a dimer. In the inbred strains, therefore, the enzyme would contain identical subunits, but the hybrid would contain, in addition, an enzyme with two dissimilar subunits.

The regulation of these isozymes of ADH appears to be accomplished by a single gene. The hybrids of the cross $Adh^F \times Adh^S$ show no association of hybrid ADH patterns with sex. When marker Ubx^{130} on the third chromosome was followed, the hybrid pattern did not segregate with

it, but it did show linkage to *Cy*, a marker on the second chromosome. Crossing-over experiments positioned the *Adh* locus between *el* and rd^S at map position 50.1 on the second chromosome.

Further experiments are exploring the characteristics of the ADH and testing a hypothesis which may be used to explain how a single gene can regulate three forms of an enzyme in the homozygous animal.

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19. Microbiology

Enzyme and Tracer Studies on Bacterial Metabolism

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19.1 CHEMICAL STRUCTURE OF THE *STREPTOCOCCUS FAECALIS* NAPHTHOQUINONE

R. H. Baum[1] M. I. Dolin

The complete chemical structure of the *Streptococcus faecalis* naphthoquinone (SFQ) has now been determined by means of nuclear magnetic resonance (NMR) spectroscopy.[2] Previous spectral and chemical work[3] had shown that SFQ is a monosubstituted 1,4-naphthoquinone containing an allyl group next to the quinonoid ring. This has been confirmed by the NMR spectra of crystalline SFQ, prepared as previously described.[4] The NMR spectra established, in addition, that the 2-substituent is a 45-carbon polyisoprenoid chain. SFQ, therefore, is 2-solanesyl-1,4-naphthoquinone. The side-chain length has been confirmed by comparison of the R_M values of isoprenologs of the SFQ and vitamin K_2 series. This comparison also indicated that both series of compounds (over the range 0 to 9 isoprene units) behave almost strictly according to partition theory during reverse-phase chromatography on silicone-impregnated paper. Thus, the effect of each isoprene unit (on the polarity) is additive, and the absence of a methyl group in the SFQ series increases the polarity of these quinones to the same extent, regardless of the length of the isoprene chain.

Crude lipid extracts of *S. faecalis* contain, in addition to the major SFQ component, small amounts of the homologs containing 35- and 40-carbon side chains. Crystallization eliminates most of the 35-carbon isoprenolog; however, the final SFQ preparation still contains approximately 10 mole % of the isoprenolog containing a 40-carbon side chain.

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19.2 THIOETHER FORMATION BETWEEN 2-SOLANESYL-1,4-NAPHTHOQUINONE AND 2-MERCAPTOETHANOL

M. I. Dolin

The key to the biological function of the 2-β-alkenyl-1,4-naphthoquinones is probably to be found in the fact that the 3 position of the molecule is

unsubstituted. In particular, the fact that *Escherichia coli* contains a 2-β-alkenyl-1,4-naphthoquinone[1] in addition to a normal complement of vitamin $K_2C(40)$ and coenzyme Q suggests that the monosubstituted 1,4-naphthoquinones have a special function that cannot be fulfilled by the 2,3-disubstituted quinones. In searching for some unique property of the monosubstituted quinones, the most striking feature that comes to mind is the ease with which such compounds form thioethers by nucleophilic substitution.[2,3]

Conditions have been described[1] under which SFQ (2-solanesyl-1,4-naphthoquinone) will react with mercaptoethanol to form a thioether which has the same spectral properties as the glutathione or mercaptoethanol derivatives of menadione. One such thioether is formed for each of the SFQ isoprenologs present in the reaction mixture. The reaction provides another colorimetric reaction for distinguishing between monosubstituted 1,4-naphthoquinones and vitamins K_1 and K_2.

The fact that a lipophilic 1,4-naphthoquinone can react to form a thioether is of interest since there is reason to believe that naphthoquinone thioethers have a biological function. Stadtman[4] has shown that a phosphatase isolated from *Clostridium sticklandii* requires a menadione thioether as cofactor. Based on this work, it has been suggested[1] that the sulfur atom of a naphthoquinone thioether may function as a phosphoryl acceptor through the formation of a transient sulfonium ion. Since it appears likely that thioethers of monosubstituted 1,4-naphthoquinones may have biological importance, several such compounds are currently under investigation.

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19.3 LOCALIZATION OF ELECTRON TRANSPORT COMPONENTS IN *STREPTOCOCCUS FAECALIS*

M. I. Dolin R. H. Baum

In order to gain further insight into the function of SFQ (2-solanesyl-1,4-naphthoquinone), the intracellular distribution of the known electron transport components of *S. faecalis* has been determined. Four-times-washed cell membranes, obtained from osmotically lysed protoplasts, contain all the SFQ and 30% of the total flavin of the cell. The levels of SFQ, flavin, and nonheme iron are, respectively, 3.5, 1.77, and 21.5 mμmole per mg of membrane protein. These values are very similar to those reported for the CoQ, flavin, and nonheme iron content of the submitochondrial preparation termed DPNH–coenzyme Q reductase.[1] Maximum release of flavin from the streptococcal membranes required treatment with proteolytic enzymes, which suggests that a portion of the flavin is covalently bound to membrane protein. Such covalently bound flavin is present in various succinic dehydrogenase preparations.[2]

Analysis of the supernatant and membrane fractions of *S. faecalis* for NADH-oxidizing enzymes has shown that the membranes contain all the cytochrome c reductase and 50% of the menadione reductase activity. The supernatant fraction contains all the lactic dehydrogenase and NADH peroxidase, 95% of the ferricyanide reductase activity, and 80% of the NADH oxidase activity. Thus, the cytochrome c reductase is the only one of the enzymes examined that is entirely associated with the cell membrane.

Further work on the structural organization and enzyme activity of the SFQ and flavin complex of the cell membrane should aid in clarifying the function of SFQ.

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20. Chemical Protection and Enzyme Catalysis

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20.1 CHEMICAL PROTECTION

D. G. Doherty Margaret A. Turner

We have previously found the protective activity of 2-aminobutylisothiourea to depend upon its optical form – the D configuration being almost twice as active as the L configuration. In conjunction with the work with glutathione it was necessary to prepare quantities of D-cysteine, and consequently we have examined the protective activity of the D isomer as compared to the L isomer. The radiation LD 50/30 days for these compounds was determined in groups of $BC3F_1$ mice that were divided in half and given 1.6 g/kg of each isomer of cysteine HCl intraperitoneally 15 min prior to X irradiation. The D isomer proved to be somewhat more protective than the L isomer, the LD 50/30 days being 1300 r for the D form and 1100 r for the L form. Ten to twenty percent survival at 1425 r was consistently obtained with the D isomer, while there were no surviving animals beyond 1275 r with the L isomer. This effect may arise from the slower metabolism of the D-cysteine, so that its effective concentration remains higher during the radiation period.

The synthetic work with the optical isomers of glutathione and isoglutathione has been concentrated on the production of sufficient quantities of the blocked isomers so that on deblocking, enough material would be on hand for mouse testing. The yields at all stages have been improved up to the removal of the sulfur and carboxyl blocking groups. Considerable difficulty has been experienced at this stage, the yields being low enough to preclude their use for preparation of the quantities necessary. These reactions are currently being studied in an effort to devise better experimental procedures.

20.2 CHROMATOGRAPHIC STUDIES OF MOUSE KIDNEY MURAMIDASE

Vu-Thi-Suu

Introduction. – In previous reports,[1–3] it has been shown that a marked increase in muramidase activity occurred in kidneys, spleen, and bone marrow of homologous and heterologous chimeras. An analytical study of this enzyme using chromatographic techniques is being made in an attempt to determine whether the increased muramidase is of donor or host origin. The first part of this investigation consists of chromatographic studies of normal mouse kidney muramidase.

Methods and Results. – Muramidase activity was determined photometrically according to the method of Shugar[4] and expressed in micrograms of hen's egg white muramidase. Protein content was determined by the Folin-Ciocalteu reagent[5] or extinction at 280 mμ.

The low concentration of muramidase in normal mouse kidney (20 μg per gram of fresh tissue) has made it essential to improve techniques for extraction of the enzyme from tissue and the preparation

of an extract suitable for chromatography. Comparative studies with different solvents have shown that a five- to sixfold increase in muramidase activity can be obtained when *M*/15 phosphate buffer, pH 7.3, is used for extraction rather than water. The literature contains conflicting reports as to the dialyzability of muramidases from various sources. Attempts at concentration of crude mouse kidney extract by dialysis in cellophane or collodion membranes led to losses as high as 90% of the total activity present. A successful procedure has been developed, utilizing a heat denaturation step to remove extraneous protein followed by absorption of the enzyme on Amberlite XE 89 or CG 50 resin in a batch process and elution from the resin in a column. The enzyme losses in this process are low, and the specific activity increased considerably. This material is currently being studied using gradient elution ion exchange chromatography.

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20.3 MURAMIDASE ACTIVITY IN LYMPHATIC MACROPHAGES OF MICE

A. J. Speece

Introduction. – The histochemical method for the demonstration of muramidase activity[1] has been improved to permit the distinction of smaller differences in activity levels and better resolution of the activity in single cells.

Results and Discussion. – The lymphatic tissues of mice treated according to the following protocol were examined and compared to normal controls. One group was given 360 mg/kg AET intraperitoneally (I.P.) prior to 900 r of x radiation. The second group received a combination of 480 mg/kg AET and 1.6 g/kg glutathione I.P. prior to 900 r. The third group received 450 r only. The fourth group was irradiated with 900 r and 50×10^6 isologous bone marrow cells injected intravenously 4 hr later. The fifth group received 900 r and 50×10^6 homologous bone marrow cells. Tissues from one mouse of each group were collected on days 1, 3, 6 through 14, 21, 42, and 56. Preparations were made from the spleens, mesenteric nodes, and portions of the small intestines bearing Peyer's patches, providing a normal and a wide variety of progressive changes in abnormal tissue conditions. A study was made of the muramidase activity of macrophages and histiocytes in these organs. These cells were found to range from very high to imperceptible activity, which seemed to be correlated with the muramidase activity of other cells in the surrounding tissues. They appeared inconspicuous by their lack of difference in activity level from the adjoining cells. This suggests that muramidase activity of macrophages and histiocytes is derived from ingestion of other cells or their fragments which are active for this enzyme. Occasional macrophages showed high muramidase activity in areas where it was otherwise lacking. In addition, a few were found which were apparently less active than adjacent cells. These exceptions provide evidence that the enzyme is acquired by ingestion rather than by simple diffusion. The extreme variability in the activity of these elements suggests that they do not initiate synthesis of this enzyme.

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21. Plant Physiology and Photosynthesis

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Plant Physiology (21.1–21.4)		**Photosynthesis (21.5)**	
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21.1 HEAT INACTIVATION OF GIBBERELLIC ACID SENSITIVITY OF LETTUCE SEED GERMINATION

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Introduction. – In lettuce seed germination, gibberellic acid (GA) produces an apparent reversal of the inhibitory effects of a wide variety of agents (e.g., darkness,[1] osmotic inhibitors,[2] far-red illumination,[3] gamma irradiation,[4] allyl trimethylammonium chloride,[5] maleic hydrazide, coumarin, 2,4-dinitrophenol, and nicotine).[6] This report indicates that heat pretreatment, in contrast to all other inhibitory treatments tried, abolishes subsequent effectiveness of GA in promoting germination at a temperature for which GA is otherwise effective in promoting germination.

Results and Discussion. – Preheating for 8 to 16 hr at 97°C, either in a gravity convection oven open to the ambient atmosphere or in a sealed vacuum oven under 0.8 atm, abolished subsequent GA sensitivity of germination at 23 ± 1°. The same concentration of GA simultaneously applied to seeds from the same lot under the same germination conditions promoted germination when germination percentages were limited by a variety of other treatments (e.g., gamma irradiation, far-red illumination, indole, mercaptoethanol, and hydrazine). The concentration of GA used, 3×10^{-4} *M*, was optimal for promoting germination percentages of unheated seeds. No concentrations (up to 3×10^{-3} *M*) increased germination percentages of seeds that had been given the heat pretreatments for 8 hr or more. Similar results were found with several lots of GA. In some cases the final germination percentage was reduced by heat, whereas in others nearly all the seeds germinated, only the rates of increase in percentage germination being reduced. In either case, subsequent GA treatment gave no apparent reversal of the inhibitory effect of the heat on percentage germination. Thus the ineffectiveness of GA on these heated seeds cannot be attributed to killing within the seed population. When the germinated seedlings were transferred to pots of soil without further GA treatment, typical GA effects were observed on growth of the seedlings that developed from the preheated seeds. This finding rules out the possibility that preheating somehow enables the seeds subsequently to destroy GA itself. Perhaps the apparent heat lability of the mechanisms underlying GA sensitivity of lettuce seed

germination may provide a clue for future understanding of the mechanism(s) by which GA breaks seed dormancy.

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21.2 USE OF RADIATION-INDUCED MITOTIC INHIBITION IN ANATOMIC STUDIES WITH GROWTH REGULATORS

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Introduction. – Gibberellic acid (GA) treatment of intact plants causes an increased stem length. A prominent aspect of this GA effect is an increased mitotic activity in the "subapical meristem," the region of the stem basal to the apical meristem and in which most or all of the stem elongation occurs.[1,2] This increased stem length is generally interpreted as resulting solely from the increased cell number.[1,2] By contrast, our studies on cell division and morphogenesis suggest that an agent that promotes growth and cell division may not necessarily promote growth by means of the increased cell division.[3] In general, one cannot choose between these two interpretations without some kind of experimental separation of growth from cell division. In this study, radiation-induced mitotic inhibition is used for such an experimental separation of the GA-induced elongation of the subapical meristem.

Results and Discussion. – Mitosis and cell division were prevented by exposing air-dry wheat grain to 700 kr of ^{60}Co gamma rays before sowing. "Gamma-plantlets," that is, seedlings from such irradiated grain, and unirradiated controls were grown with or without GA. Absence of cell divisions in gamma-plantlets was shown by equality of cell numbers in longitudinal rows of the subapical meristems with the corresponding cell numbers in embryos of unsown grain. Plants treated with GA have significantly longer subapical regions than water controls, whether they have grown with (unirradiated) or without (gamma-plantlets) cell divisions. In the unirradiated plants the GA effect on elongation of the subapical region is accompanied by a greater number of cell divisions. Since in gamma-plantlets GA similarly promotes elongation of the subapical region, except that there are no cell divisions, these findings cast doubt upon the interpretation that GA promotes stem elongation by means of increased cell division. This study illustrates how radiation-induced mitotic inhibition can be used to investigate in a distinct anatomic region the relation of cell division to the chemical regulation of growth.

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21.3 METABOLIC CONTROL IN MAIZE EMBRYOS[1]

Ann Oaks

When a seed germinates, synthesis of macromolecules in the embryo and degradation of storage products in the endosperm are initiated. In maize, anatomical[2] and chemical[3] changes associated with germination are observed first in the embryo and then proceed to the endosperm. This is a clear example of reactions in one plant part inducing changes in another plant part. The interactions between the two opposed regions in the germinating seed can be studied easily because: (1) the growing region (the embryo) and the senescing region (the endosperm) are distinct, (2) the reactions involving growth and senescence can be initiated simply by the addition of water, and (3) the two regions can be separated and maintained for a time under sterile conditions. Thus, one can ask whether a specific stimulus from the embryo controls the onset of the degradation of the storage products in the endosperm. Conversely, one can ask whether the transport of metabolites from the endosperm imposes a regulation on the

development of biochemical reactions within the embryo. Using whole plants, isolated plant parts, and plant homogenates, we have studied two aspects of this problem: (1) degradation of storage nitrogen in the endosperm and the transport of these degradation products to the embryo, and (2) possible controls exerted on the developing embryo by this transported nitrogen.

Loss of Nitrogen from the Maize Endosperm. – The best known hydrolytic enzymes of the endosperm, α-amylase, β-amylase, and protease, show characteristic increases with germination, and, in detached endosperms, each is stimulated by gibberellic acid (GA).[4] There are, however, distinct differences between the enzymes involved in the hydrolysis of carbohydrate and those involved in the hydrolysis of protein. The embryo factor which appears to be essential for the degradation of storage carbohydrate does not appear to be critical for proteolysis. When maize seeds were grown in a salts solution, there was a loss of nitrogen which was apparent within 12 hr. When a synthetic mixture of 15 L-amino acids known to be released by the endosperm[5] was also included in the medium, the loss of nitrogen was delayed for approximately 40 hr. Since an independent supply of organic nitrogen to the seedling prevents the loss of nitrogen from the endosperm, we conclude that the movement of soluble nitrogen out of the endosperm was sufficient to trigger proteolysis. We suspect that on germination the soluble amino acids in the embryo are used in the synthesis of protein, thus establishing a concentration gradient of amino acids in the seedling. When a critical low level of a particular amino acid is reached in the endosperm, the reactions leading to the degradation of storage protein are triggered.

Synthesis of Leucine in Maize Embryos. – When maize embryos are detached from their endosperms and grown in sterile culture, there is a lag of several days before a normal increase in protein nitrogen is observed. This lag is overcome by the addition of an endosperm mixture of amino acids to the medium.[5] From the extensive work with bacterial systems[6] it was reasonable to postulate that at the time of dissection the enzymes which synthesized amino acids in the embryo were in an inhibited state, due to the ready supply of amino acids from the endosperm. To test this hypothesis, the enzymes involved in the biosynthesis of leucine in maize embryos were examined. The specific activities of at least two of the leucine biosynthetic enzymes (the α-hydroxy-β-carboxyisocaproic acid dehydrogenase and the final transaminase) increased with time after germination. If this control of enzyme activity were at the level of repression of enzyme formation by the ultimate end product (leucine), then limiting the leucine pool should result in an increase in the leucine biosynthetic enzymes. Three reactions [α-hydroxy-β-carboxyisocaproic acid dehydrogenase, the conversion of β-carboxy-β-hydroxyisocaproic acid to α-ketoisocaproic acid (isomerase plus dehydrogenase), and the final transaminase] showed lower specific activities when leucine was absent. Thus, the typical bacterial-type derepression, in which each enzyme of a given pathway is affected, was not apparent in these embryos.

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21.4 REDUCTION OF AIR TURBULENCE AND TEMPERATURE GRADIENTS IN PLANT GROWTH CHAMBERS BY MEANS OF SMALL-HOLE DIFFUSER WALLS

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Introduction. – The purpose of a controlled-environment chamber is to have at a given time a uniformity of a given temperature and a given humidity. In many cases, especially with growing plants, it is essential to achieve this goal without excessive wind velocities and associated air turbulence within the chamber. The problem of temperature gradients in plant growth chambers is especially difficult when high-intensity illumination is used. To date, there has been no satisfactory solution to the problem of passing through

the chamber the large volumes of air necessary to maintain control conditions without producing point air velocities and associated turbulence superoptimal for plant growth. This report describes a simple method for uniformly introducing sufficiently large volumes of preconditioned air into growth chambers while producing very small point velocities and little turbulence in all regions of plant growth chambers farther than 1 or 2 in. from the walls.

Results and Discussion. – In a large (116 × 62 × 82 in.) illuminated commercial growth chamber, there were approximately 20°F vertical temperature gradients and horizontal gradients of 5 to 10°F even when the air flow and associated turbulence were excessive. Installation of a plenum containing two 112 × 47.5 in. stainless steel perforated diffuser walls, each 4 in. from the two original longitudinal walls, provided an air plenum for the preconditioned air. Small, evenly spaced holes ($\frac{1}{16}$ in. in diameter, $\frac{1}{8}$ in. between centers) in the diffuser walls provided sufficient resistance to air movement into the newly modified chamber to maintain a uniform, constant pressure differential across the diffuser walls. Consequently, a small jet of high-velocity air moves into the chamber through each of the small holes and expands conically. The energy associated with these numerous symmetrical and evenly spaced air currents is rapidly and evenly dissipated within a few inches of the diffuser walls, so that there is negligible turbulence within almost the entire growth room. The volume of air moving per unit time through the room is only slightly reduced and is sufficient to maintain with negligible turbulence a hitherto unrealized uniformity of temperature. Even with high-intensity illumination (up to 3600 ft-c) the vertical temperature gradient was 1.5°F with horizontal gradients less than 0.5°F. Without illumination, there were no detectable horizontal or vertical temperature gradients.

The same principle was applied in modifying a small (22 × 20 × 17 in.) plant growth chamber in which preconditioned air was introduced through a vertical row of nine $1\frac{1}{2}$-in.-diam holes on each of two opposite side walls. Although temperature and humidity gradients had been satisfactory, the turbulence was excessive and deleterious to plant growth. Installation of the same perforated material over each of the rows of air inlets reduced the air turbulence to negligible levels. In both types of chambers modified by this simple method, it was not necessary to rework the control systems or otherwise produce extensive modifications of basic design. Because the principle involved can be applied in relatively simple modifications of existing growth chambers, as well as in the design of new chambers, it gives promise of greatly improving the performance of plant growth chambers.

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21.5 PHOTOSYNTHESIS

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Photosynthesis is the only major process by which energy is injected into the biological cycle. The work of Calvin, Benson, and co-workers has elucidated the dark reactions by which carbon dioxide is converted to sugars. However, the initial energy-conversion steps are still unclear. How does the absorption of light by chlorophyll lead to the production of those high-energy chemical substrates, adenosine triphosphate and reduced pyridine nucleotide, which initiate the enzyme chemistry of the Calvin cycle? This question is of more than theoretical importance; at the present growth rate of the earth's human population, it will be only about 50 years before the number of humans is too large to be fed by the amount of land which can be cultivated on the earth. It is clearly imperative to understand photosynthesis to at least an extent which would allow the process to be duplicated artificially.

In 1951 it was discovered, in this laboratory, that photosynthetic organisms emit a long-lived dim glow from chlorophyll. Studies of this delayed light led to the electrical experiments on dried plants which originally implicated a solid-state physical process in photosynthetic energy conversion. For the past three years we have been engaged in an intensive study of the details of the delayed-light emission of living plants, because this emission affords a means of studying electron transitions over a wide time range within the functioning photosynthetic apparatus.

One of our main aims has been to extend measurements of the delayed-light emission to very short times. We have now developed a Kerr cell apparatus which is capable of measuring the emission to times as short as 5×10^{-6} sec from the end of a flash of exciting light. With this new measurement, we now have studied the delayed-light decay over nine orders of magnitude in time, our longest measurement being at 6×10^{3} sec. Although the precise physical mechanism of delayed-light emission remains as unclear as the mechanism of photosynthetic energy conversion, this long decay curve constitutes evidence from living plants that solid-state physical mechanisms are involved in photosynthesis.

In 1962 we found the presence of more than one photoreaction to be reflected in the delayed-light emission. This conclusion is in agreement with other lines of evidence from several other laboratories. It became clear that in order to study the physical process of energy conversion, we would have to simplify the biological system by studying one photoreaction at a time and by controlling the enzyme chemistry which accepts electrons (or holes) from chlorophyll. We have now achieved these aims for delayed-light measurements from whole cells in the range from 1 to 100 msec.

We have been able to show that from 1 to 100 msec, only one photoreaction is emitting the delayed light. About a year ago, we studied the millisecond delayed-light emission from a mutant photosynthetic bacterium, in collaboration with R. C. Clayton. We found that the emission of delayed light depends on the presence of functional photosynthetic reaction centers within the chlorophyll units. With this information we were able to study a group of mutants of algae whose reaction centers for one photoreaction are either missing or nonfunctional, while the other photoreaction is left intact. These mutant strains of *Scenedesmus obliquus* were isolated by N. I. Bishop, Department of Botany, Oregon State University, Corvallis, Oregon, who made them available for our use. We found that the mutants in which the short-wavelength photoreaction was blocked (these mutants cannot perform the quinone Hill reaction, but are able to photoreduce CO_2 in the presence of hydrogen gas) did not emit detectable intensities of delayed light. This observation implies that in this time range all delayed light is emitted from the short-wavelength, oxygen-evolving photoreaction, since the long-wavelength photoreaction was intact, but no delayed light was observed. Furthermore, mutants in which the long-wavelength photoreaction was blocked (these mutants are able to perform the quinone Hill reaction, but do not contain P700 and are unable to photoreduce CO_2 in the presence of hydrogen gas) emitted up to eight times the intensity of delayed light emitted from the wild type. The kinetics of delayed-light emission from these mutants were very "flat" compared with the wild type. We interpret these changed kinetics as implying that the enzyme chain which links the two photoreactions was completely reduced and therefore was unable to accept electrons from the short-wavelength photoreaction (which emits delayed light), due to the long-wavelength photoreaction being unable to accept electrons from the enzyme chain. The delayed-light emission was therefore increased and extended over a longer period, since normal electron flow into the enzyme chain was effectively blocked.

We tested this hypothesis by introducing an electron acceptor, *p*-benzoquinone, into the whole cells. Under the conditions we used, this chemical has little effect on the delayed-light emission of the wild type, but it does allow both the wild type and the mutant to perform the Hill reaction (photoevolution of oxygen with concomitant reduction of quinone). When the mutant was performing the Hill reaction, its delayed-light emission was changed to that of the wild type. That is, the intensity of emission was reduced, and the kinetics were normalized. This is the first direct evidence that delayed light is modulated by the rates of enzymatic reactions in the photosynthetic electron transport chain.

With our present knowledge that the millisecond delayed-light emission is from only the short-wavelength photoreaction, and that the "flat" decay kinetics result from a block in electron flow out of this short-wavelength photoreaction, we are in a position to interpret more precisely the meaning of our earlier measurement of a 0.5-ev activation energy for the delayed-light emission of cells poisoned with CMU [3-(*p*-chlorophenyl)-1,1-dimethylurea]. Since delayed light is emitted only from the short-wavelength photoreaction, we can say that the 0.5-ev activation energy must be

associated with the short-wavelength photoreaction. The CMU-poisoned cells show a decay which is even more "flat" than that of the mutant cells. Since the "flat" decay kinetics apparently result from a block in electron flow, we can say that the CMU must be blocking electron flow and that the 0.5 ev represents a physical process within the chlorophyll system rather than some enzymatic process which is modulating the delayed light.

References

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22. Biophysics

Radiation Biophysics (22.1–22.5)		Molecular Biophysics (22.6–22.9)	
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22.1 MEASUREMENTS OF LOW CONCENTRATION OF FREE RADICALS

M. L. Randolph

Introduction. – The significance of free radicals in biological processes depends on both the free radical species and their concentrations, which are often at the border line of measurement. The estimation of a concentration is usually reached by second-integral or first-moment calculations from the curve of (derivative of absorption) vs (magnetic field) presented by an electron-spin-resonance spectrometer. We have investigated, in terms of experimental limitations and errors, the precision of such concentration estimates and some techniques for the reduction of errors.

Results and Discussion. – In Fig. 22.1.1, difficulties in the input data for concentration calculations are depicted in exaggerated form. The experimental approximation (da/dx) to the true derivative of the absorption (dA/dh) becomes:

$$\frac{da}{dx}=\frac{dA}{dh}-\left(\frac{dA}{dh}\right)_{\min h}+\alpha+\beta\left(X+\frac{S}{2}+\gamma\right).$$

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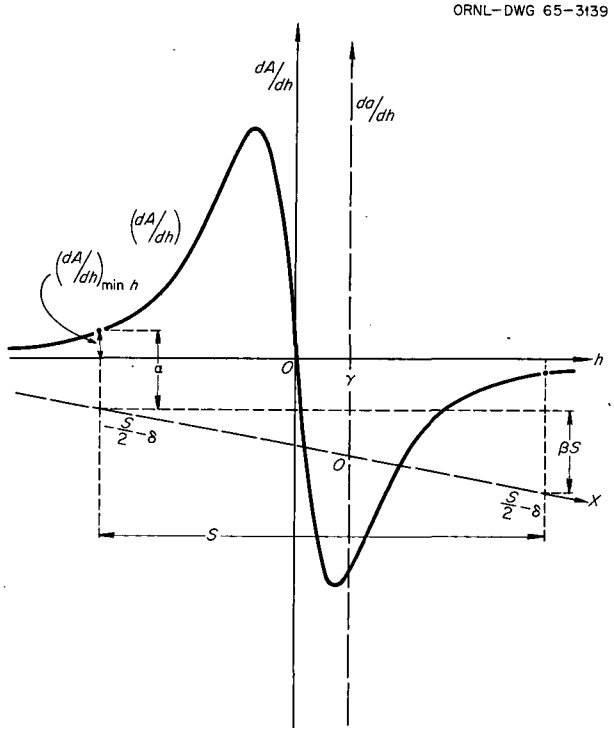

Fig. 22.1.1. Schematic Presentation of Errors in Measurement of ESR Curve for Concentration Estimation. The solid lines represent the true axes, and the dashed lines the experimental axes.

The finite scan, S, or duration of a measurement precludes measurement of the tails of curves and introduces an approximation that the initial value of (dA/dh) is zero. Errors in ordinate measurements are the zero error, α, and the possibility of base-line drift, shown here as culminating in a value βS, since, under at least some instrumental conditions favorable for concentration measurements, there is a base-line drift logically and experimentally nearly linear with the magnetic field. Errors in centering the scan on the center of the absorption curve are represented by γ and δ. For the most common absorption curve shapes (Gaussian and Lorentzian), exact formulas for the effects of these aberrations in first-moment and second-integral methods have been nearly completed. For spectra with equal widths at half maximum absorption, data from equal scan widths, S, yield more precise values for Gaussian than Lorentzian shapes. The first-moment method reduces dependence on the experimental error α, but, using a simple, previously suggested correction method for the first-integral calculation, the dependence on α can be eliminated and other errors greatly reduced.[1] For the first-integral method with a given scan, S, the precision is improved by placing the center of the pattern nearer the end than the start of the scan. Experimentally, the value of β may be reduced by dual cavities run in out-of-phase modulation[2] and by a recently devised feedback circuit.

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22.2 ULTRAVIOLET MICROBEAM IRRADIATION OF NUCLEUS AND CYTOPLASM OF *AMOEBA PROTEUS*

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Introduction. – For many cells, division delay and death following treatment with ionizing radiation are due largely or completely to effects on the nucleus.[3] Several reports, however, involving both ionizing and ultraviolet radiation, indicate an important role of cytoplasm, either in induction of the damage leading to these effects or in recovery of the cell from the damage.

In the present experiments, starved daughter cells (probably all in interphase) of *Amoeba proteus* were flattened to 20- to 30-μ thickness and then irradiated with microbeams of polychromatic (2400–3400 A) ultraviolet radiation (uv). This technique enables virtually complete irradiation of nucleus or cytoplasm, and it permits the normal nucleus-cytoplasm interactions to occur after irradiation. It avoids most of the weaknesses of experiments done on unflattened cells,[4] fragments of cells,[5] or nuclear transplants.[6] The information obtained can, therefore, be applied to the question of *the relative roles of nucleus and cytoplasm in whole-cell irradiation effects.*

It is reasonably certain that photoreactivation (PR) operates only on damage to nucleic acids. Nuclear damage clearly can be photoreactivated, but the case for cytoplasmic PR is equivocal. Skreb and Errera[5] found PR of uv-induced reduction of the life span of enucleate halves of *A. proteus*, but this does not tell us if PR would be found for effects on whole cells caused by cytoplasmic irradiation.

Methods. – Techniques involving use of a 50× objective have been described in an earlier preliminary report.[7] The present report includes results from roughly twice as many data with the 50× objective as were then available. Cytoplasmic irradiations with this objective were done by exposing the amoeba cytoplasm to four nonoverlapping circular fields, a technique that permitted irradiation of only about 60% of the cytoplasmic area. In addition, results are here presented that were obtained with a 20× objective, which permitted irradiation of the entire cytoplasm of an amoeba without irradiation of the nucleus.

Results. – The present complete data with the 50× objective agree only qualitatively with the earlier preliminary data. Whereas we earlier reported that nucleus and cytoplasm showed roughly equal sensitivity to a given uv *dose* (energy incident per unit area) for either division delay or killing, the more complete data show that the cytoplasm sensitivity may vary from about 0.5 to 0.1 that of the nucleus when division delay is the criterion, and is roughly 0.1 that of the nucleus when killing is the criterion. Exact comparisons of nuclear to cytoplasmic sensitivity are difficult,

since the curves of the logarithm of division capability vs dose are concave upward for the cytoplasm but linear for the nucleus. Effects obtained by irradiation of the cytoplasm show great variability. This is, undoubtedly, largely due to the fact that the amoeba generally rejects those parts of its cytoplasm that are seriously damaged. This rejection results in a smaller cell, which must then grow to a normal size before it can again divide. Thus, since the total radiation field is constant in area, the amount of division delay induced will, among other things, be a function of the size of the cell irradiated. Because of this ability, it is most difficult to kill an amoeba by irradiation of cytoplasm.

Irradiations where one of the four cytoplasmic fields includes the nucleus yield a sensitivity for division delay about twice that for nuclear irradiation but roughly the same for killing. The killing result is as expected, since cytoplasmic damage contributes so little to killing. The division-delay result, however, shows, in agreement with our earlier conclusion, that the cell is more sensitive when both nucleus and cytoplasm are irradiated than would be expected, thus indicating that the nucleus and/or cytoplasm possess a recovery potential for damage to the other organelle. This is consistent with reports of such a recovery function for the cytoplasm of another amoeba, *Pelomyxa illinoisensis*.[8]

Division delay is highly photoreactivable (photoreactivable sectors of about 0.5) when induced by either nuclear or cytoplasmic damage. Killing is slightly photoreactivable when induced by nuclear damage. We have not yet been able to test for PR of killing due to cytoplasmic damage.

Experiments with the 20× objective, where the entire cytoplasm is irradiated, yield different results. For division delay, the cell is found to be three times as sensitive to cytoplasmic damage as to nuclear damage, and six times as sensitive to whole-cell damage as to nuclear damage. It is clear from these data that irradiation of only 60% of the cytoplasmic area yields a false impression of the importance of cytoplasm, an effect apparently due to the remarkable ability of this cell to reject damaged tissue. The data for whole-cell irradiation confirm the conclusion that the nucleus and/or cytoplasm possess a recovery potential for damage to the other organelle. These experiments also show that, even when the entire cytoplasm is irradiated, photoreactivability is high.

Conclusions. – Taken together, these data show that, when a whole flattened *A. proteus* is irradiated with uv, damage to cytoplasm and damage to nucleus contribute very roughly equally to the observed division delay. This shows that cytoplasmic damage in this cell is very important. However, it must be borne in mind that division delay resulting from nuclear damage probably arises from a fundamentally different cause (probably genetic) than that resulting from cytoplasmic damage (probably the need for the cell to regenerate cytoplasm), a situation that can lead to disparate results in all but the most carefully conducted experiments and which demonstrates the inadequacy of experiments on unflattened amoebae, where both the nucleus and part of the cytoplasm are partially shielded from the incident radiation.

Cell killing by uv appears to be caused primarily by nuclear damage.

These experiments are consistent in showing a recovery function of the nucleus and/or the cytoplasm for the other organelle.

These experiments also demonstrate unequivocally, and for the first time, that damage to a whole cell caused by cytoplasmic irradiation can be photoreactivated. This finding also strengthens the earlier indication[5] of the presence of photoreactivable sites in the cytoplasm. Unfortunately, it does not prove that cellular ribonucleic acid (RNA) is photoreactivable, since the presence of deoxyribonucleic acid (DNA) components in cytoplasm is becoming daily more evident for many types of cell, and has been clearly demonstrated in the case of *A. proteus*.[9]

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22.3 ULTRAVIOLET PHOTOPRODUCTS IN 5-BROMOURACIL–SUBSTITUTED DNA OF *ESCHERICHIA COLI* 15T⁻

M. B. Lion R. B. Setlow

Introduction. – The irradiation product of the dinucleotide TpBU was shown by A. Haug[1] to be a debrominated dimer of uracil and thymine containing a cyclobutene ring. The possible formation of this compound by irradiation in vivo seemed of interest. Smith,[2] by irradiation in vivo of BU-substituted DNA of *Escherichia coli* B/r and *Enterococcus stei,* showed that the nature of the photoproducts depended on the organism. Using only singly labeled cells, he could exclude only indirectly the formation of a bromouracil-thymine derived dimer.

Methods. – Cells doubly labeled with ^{14}C-thymine and ^{3}H-bromouracil were prepared. Seventy to seventy-five percent of the thymine residues in DNA were substituted by bromouracil. They were either irradiated and then the DNA was isolated or the DNA was isolated first and was then irradiated in vitro in 0.01 *M* Tris buffer at pH 8.0 with $\sim 2 \times 10^4$ ergs/mm^2 of 2800 A. The DNA was hydrolyzed and chromatographed in butanol–acetic acid–water, and strips of the chromatogram were counted in a scintillation counter.

Results. – Irradiation in vivo gave rise to four tritium peaks (derived from BU) and two ^{14}C peaks (derived from thymine). No peak contained both labels, which excludes the formation of the compound suggested by Haug.[1] The two thymine-derived products, when eluted and reirradiated in solution, gave rise to free thymine. They are probably TT and UT dimers. None of the bromouracil-derived products could be split in this way. Their R_f's are 0.10, 0.19, 0.32, and 0.49.

Irradiation of native BU-substituted DNA gave qualitatively the same results but showed a somewhat higher total photochemical conversion than irradiation in vivo. Quantitatively, the peak at 0.49 was much higher and was identified as uracil. The product at $R_f = 0.32$ split into at least two components in other solvent systems. Neither seems to be identical with uridine.

In the presence of 0.01 *M* cysteamine, the amount of uracil formed from the irradiation of native DNA was decreased by 50%, and two of the other products were considerably increased; however, the total photochemical conversion remained exactly the same. Thus, our system behaves differently in presence of cysteamine than does the T1 phage system described by Rupp and Prussoff.[3] Also, cysteamine does not protect BU-substituted *E. coli* 15T⁻ cells against killing by uv light.

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22.4 COMPARISON OF THE UV ACTION SPECTRA FOR CELL KILLING OF *ESCHERICHIA COLI* B/r AND B_{s-1}

M. B. Lion R. B. Setlow

While the high sensitivity to uv light of *Escherichia coli* B_{s-1} as compared to B/r is probably due to absence of dark repair,[1] it seemed, nevertheless, of interest to test whether the primary target to uv light was the same in both organisms. The uv action spectra for killing have, therefore, been compared in these cells.

The slopes of the straight-line portions of the survival curves on semilog plots have been used as parameters defining sensitivity. With this definition the action spectra of both strains turned out to be identical within experimental error – the sensitive strain being approximately 100 times more sensitive than B/r at all wavelengths tested (2300, 2380, 2480, 2650, 2805, 2900, and 2967 A). Each of the survival curves of B/r had a considerable shoulder. The extrapolation numbers of these survival curves varied bimodally with wavelength.

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22.5 LONG-LIVED NEUTRON ACTIVATION OF ACCELERATOR

J. L. Hosszu M. L. Randolph

Introduction. – Prolonged high-level operation of an accelerator to produce 14-Mev neutrons by the prolific $T(d,n)^4He$ reaction results in both short- and long-lived nuclear activation of the target region and shielding walls. We have determined the long-lived radioproducts by gamma spectral analysis after a 2-hr high-intensity irradiation, and by radioactive decay measurements, using a scintillation counter and a multichannel analyzer.

Results and Discussion. – The products, their relative initial intensities, half-lives, and probable nuclear reactions from which they come are given in Table 22.5.1. Near the target assembly, which was predominantly yellow brass, the intensity was at least an order of magnitude higher than that near the walls. All the radioactive products listed emit high-energy gamma rays. The wall product ^{27}Mg was not observed in this experiment because of its short half-life, but approximately this decay rate has been observed previously with ion chamber measurements; also, from the comparable cross sections for $Al(n,p)$ and (n,α), ^{27}Mg must be present. No products of neutron irradiation of the Pt-backed Zr target were observed.

22.6 PROPERTIES OF PYRIMIDINE PHOTOPRODUCTS IN DNA

R. B. Setlow W. L. Carrier

Ultraviolet irradiation of DNA leads to the formation of pyrimidine dimers of the form $\widehat{TT}$, $\widehat{CT}$, and $\widehat{CC}$.[1] We have investigated the relative frequency of these dimers as functions of incident wavelength, dose, and base composition. At all doses between 280 and 240 mμ, the ratio of $\widehat{CT}$ to $\widehat{CC}$ is 2 in *E. coli* DNA. At low doses the ratio of $\widehat{TT}$ to $\widehat{CT}$ is 1.2 at all wavelengths, but at doses above 5×10^3 ergs/mm^2 the ratio depends strongly on wavelength because cytosine-containing dimers are split about ten times more rapidly than $\widehat{TT}$ and hence reach their steady-state values at lower doses.

The relative amounts of $\widehat{CT}$ and $\widehat{CC}$ depend, as expected, on base composition. The dependence is such that the sum of $\widehat{TT}$, $\widehat{CT}$, and $\widehat{CC}$ is proportional to the (A + T) content of the DNA. It has been found that the uv sensitivity of microorganisms is proportional to (A + T).[2]

We have used the distinctive photochemical properties of $\widehat{CC}$ and $\widehat{UU}$ ($\widehat{CC}$ is split by 240 mμ more rapidly) and the fact that the U arising from dimer splitting in a C-labeled DNA is easily

Table 22.5.1. Target and Wall Activation by 14-Mev Neutrons

Region	Radioproduct	Relative Initial Intensity of Gamma-Ray Emission	$t_{1/2}$ (days)	Probable Modes of Production
Target	^{64}Cu	10.0	0.53	$^{65}Cu(n,2n)$, $^{64}Zn(n,p)$, $^{63}Cu(n,\gamma)$
	^{59}Fe	2.6	45	?
	^{65}Ni	1.2	0.11	$^{65}Cu(n,p)$, $^{68}Zn(n,\alpha)$
	^{65}Zn	0.6	24.5	$^{64}Zn(n,\gamma)$, $^{66}Zn(n,2n)$, $^{65}Cu(n,\beta^-)$
Wall	^{24}Na	High	0.625	$^{27}Al(n,\alpha)$
	^{27}Mg	High	0.06	$^{27}Al(n,p)$
	^{40}K	Low	$>10^{11}$	$^{39}K(n,\gamma)$
	^{47}Ca	Low	4.8	?

detected to show that cytosine dimers are deaminated:

$$\widehat{CC} \xrightarrow{\text{heat}} \widehat{UU} \xrightarrow{240\ m\mu} U + U\ ,$$

$$\widehat{CT} \xrightarrow{\text{heat}} \widehat{UT} \xrightarrow{240\ m\mu} U + T\ .$$

Deamination in native DNA at pH 7 is complete in 5 min at 100°, but only goes about 30% in 60 min at 60°. In denatured DNA, the deamination rate is higher and is comparable to that observed in the polymer dI:dC.[1]

All types of pyrimidine dimers in DNA are split in vitro by photoreactivating enzyme plus 365 mμ radiation.[3] The relative rates of splitting are $\widehat{TT}$: 1; $\widehat{CT}$: 0.5; $\widehat{CC}$: 0.3. We have also found that the excision rates in vivo in cells that have repair mechanisms are the same for $\widehat{TT}$ and $\widehat{CT}$ and that excision in a strain of *E. coli* requiring T, U, and amino acids is unaffected by their absence from the growth medium.

Cell-free extracts of *M. lysodeikticus* are able specifically to excise dimer-containing acid-soluble oligonucleotides from irradiated DNA.

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22.7 ULTRAVIOLET PHOTOPRODUCTS IN BACTERIAL SPORES

J. E. Donnellan, Jr. R. B. Setlow

Introduction. – Bacterial spores are much more resistant to uv irradiation than vegetative cells. It was felt that the high resistance might arise from the peculiar physical state of the spores – a state in which pyrimidine dimers are *not* formed by uv irradiation. Initial experiments[1] showed, however, that uv irradiation of spores produced large amounts of a product formed from thymine. We have characterized this photoproduct and shown that it is not $\widehat{TT}$.

Results and Discussion. – Spores of *B. megaterium* in suspension, labeled with ^{3}H-thymidine, were irradiated with monochromatic radiation. The DNA was extracted and the acid hydrolysate chromatographed in butanol–acetic acid–water. Up to 30% of the thymine label appeared in an unknown product (X) whose R_f was 0.38. No activity was found in the $\widehat{TT}$ region; 2% was in the $\widehat{UT}$ region. (We have not yet identified this product.) The amount of product X saturated at a value of 30% with 5×10^4 ergs/mm^2 of 2650 or 2800 A. Subsequent irradiation of spores by 2390 A failed to change the product, and irradiation of isolated X in solution did not alter it. This result eliminates the possibility of a photosteady state as the cause for the 30% saturation levels. A dose, 1000 ergs/mm^2, at which there is 100% survival, yields 1.3% of the thymine in product X. Thus there is one X every 250 to 500 nucleotides, or approximately 50,000 per bacterial genome.

It is possible to produce product X by irradiating dry DNA in the presence of calcium dipicolinate – a compound that is ~10% of the dry weight of spores. The product interferes with the enzymic hydrolysis of DNA. Attempts are being made to identify X by observing the photoproducts obtained from dry synthetic polydeoxyribonucleotides.[2]

Conclusion. – Normal thymine dimers are not produced by uv irradiation of bacterial spores, but other photoproducts are produced to a large extent. In order to survive, the spores must have a mechanism to recognize these photoproducts and remove them from the bacterial genome during differentiation of the spores into vegetative cells.

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22.8 LOSS OF RADIOACTIVITY FROM IRRADIATED BACTERIA

Carolyn R. Shaffer R. A. McGrath

Introduction. – DNA is degraded to acid-soluble material and lost from bacterial cells after x-ray

exposure.[1-3] The purpose of this study was to determine autoradiographically whether, after x irradiation, material is lost by all cells or if a fraction of the population loses most of its DNA while the remainder loses none.

Results. – A radiation-resistant (B/r) strain of *E. coli* was labeled with ^{3}H-thymidine, exposed to x rays, and then incubated for 50 min at 37°C. Samples were taken before and after the incubation period. Acid-soluble material was extracted from cells, and autoradiograms were prepared and scored (7-day exposure, Kodak NTB-2 emulsion). Results are summarized by the distribution curves shown in Fig. 22.8.1. Figure 22.8.1*a* and *b* shows a comparison of grain distributions of unirradiated cells before and after incubation for slightly more than one cell generation. During this time the average grain number per cell is reduced by about 60%. After exposure to 10 or 50 kr, most cells were 2 to 2½ times longer than nonirradiated controls and had apparently not divided. The average grain number shifts from 15.4 to 11.1 after 10 kr (Fig. 22.8.1*c*) and from 15.4 to 8.6 after 50 kr (Fig. 22.8.1*d*), representing losses of 28 and 44% respectively. With B_{s-1}, a radiation-sensitive strain, losses of 65% (10 kr) and 77% (50 kr) were observed. Since after 50 kr few cells are found in the zero class of the distribution, it is clear that all cells have lost a similar fraction of their original radioactivity. Since 75% of the B/r cells exposed to 10 kr can survive and subsequently form viable colonies, the results suggest that these survivors, which have lost 28% of their DNA, must either repair or replace the lost material or manage to continue functioning after an appreciable loss of genetic material.

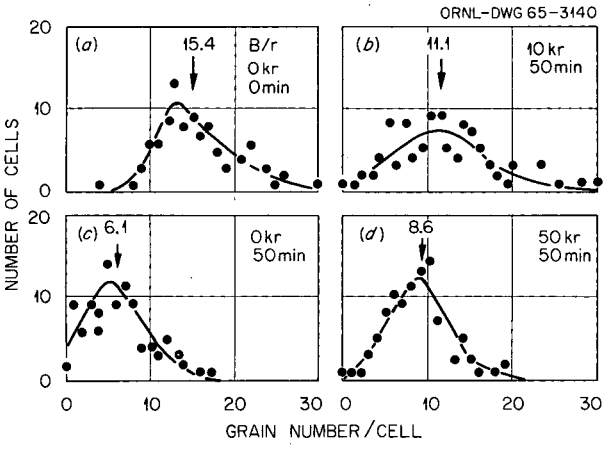

Fig. 22.8.1. Distribution Curves of Silver Grains in Autoradiograms of ^{3}H-Thymidine-Labeled *E. Coli* (B/r) After 0, 10, or 50 kr of X Rays.

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22.9 DNA TRANSFER IN BACTERIAL CONJUGATION

L. G. Caro J. Gross[1]

Introduction. – There are two main theories for the transfer of DNA from Hfr to F⁻ in bacterial mating: one, proposed by Jacob and Brenner,[2] according to which a replication of the chromosome occurs simultaneously with mating, a newly replicated copy being transferred; the other, proposed by Bouck and Adelberg,[3] involving the transfer of a finished chromosome, with the condition that a round of DNA replication must be completed before transfer can start. Since there is no overwhelming evidence in favor of either model, we attempted to test some of the predictions they allow.

Results. – Crosses were made between Hfr CR34 (λ) (thy⁻ L⁻ Str^S) and F⁻ C-416 (ade⁻ arg⁻ try⁻ met⁻ λ^R Str^R). The DNA synthesized in the Hfr was labeled with thymine-^{3}H. DNA synthesis was prevented in the F⁻ by adenine starvation before and during the cross. The analysis of the amount of DNA transferred was made by quantitative autoradiography after separation, by shaking, of the couples. The distinction between F⁻ and Hfr was by morphology (the F⁻ is spherical, the Hfr rod shaped) and by differential thymine uptake (DNA synthesis continues normally in the Hfr and is much higher than DNA transfer to the F⁻). Zygotic induction in the F⁻ of the λ prophage was used as a measurement of efficiency of transfer of genetic markers.

Kinetics of DNA Transfer. – The Hfr is labeled for seven generations, thymine-^{3}H being also

present during mating. Regardless of the model, DNA transferred will be labeled on both strands. In this system, transfer is initiated rapidly and remains linear up to 50 min. The rate of transfer thus determined is 9.8 μ/min, that is, 112 min for a chromosome 1100 μ long. This is in good agreement with genetic data (100 to 110 min).

Chaser During Transfer. – If, in the same type of experiment as the immediately preceding one, a chaser of cold thymine is introduced 20 min after contact, the subsequent transfer of label is decreased by half. Analysis of grain counts shows that the same proportion of F^- cells is labeled at 20 and 50 min (last time used). The rate of transfer of lambda is the same with and without chaser. It seems, therefore, that half of the DNA transferred is newly replicated.

Pulse of Label During Transfer. – If unlabeled Hfr's are mated to F^- and a pulse of thymine-^{3}H is introduced at 20 min, when transfer is taking place in a large majority of the cells, the F^- becomes labeled, the amount of label being proportional to time. The rate of labeling is consistent with the notion that a DNA molecule labeled on one of its strands is being transferred. A chaser of cold thymine added to the mixture stops further incorporation instantaneously.

Discussion. – These experiments rule out the possibility that a finished chromosome only is transferred. They do indicate that a replication of the DNA takes place in the Hfr immediately previous to transfer and that only one of the two newly made copies thus made (assuming a normal system of replication) is transferred. The pulse experiment shows that this replication proceeds at a rate equal to the rate of transfer, that is to say at one-third of the normal rate of chromosomal replication. These findings agree with the main aspects of the replicon model of Jacob and Brenner, which are concerned with bacterial conjugation. Another possible but unlikely explanation for these results, now being tested, is that the replication takes place in the F^-, using adenine and thymine transferred through the mating bridge.

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ORNL-3267	Period Ending February 15, 1962
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ORNL-3427	Period Ending February 15, 1963
ORNL-3498	Period Ending August 15, 1963
ORNL-3601	Period Ending February 15, 1964
ORNL-3700	Period Ending August 15, 1964