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SEMIANNUAL PROGRESS REPORT

FOR PERIOD ENDING JULY 31, 1965

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BIOLOGY DIVISION

SEMIANNUAL PROGRESS REPORT

For Period Ending July 31, 1965

Alexander Hollaender, Director

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NOVEMBER 1965

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Publications and Lectures

PUBLICATIONS

During this report period (February 16–July 31, 1965), 343 papers, abstracts, and book reviews by members of the Biology Division have been published or are in press. This total compares with 362 for the February 16–August 15 period in 1964 and with 348 for the August 16, 1964–February 15, 1965, period. Listed below are the 93 published papers, reports, small conference booklets, and books edited by Division members for the current period. The 64 published abstracts, 5 published book reviews, and 181 items in press are not listed.

The Proceedings of the Eighteenth Annual Biology Research Conference on **Hormonal Control of Protein Biosynthesis** will be published by Wistar Institute of Anatomy and Biology as an October supplement to the *Journal of Cellular and Comparative Physiology*.

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Adler, H. I., and Alice A. Hardigree	Growth and division of filamentous forms of *Escherichia coli*	*J. Bacteriol.* **90**, 223–26 (1965)
	Postirradiation growth, division, and recovery in bacteria	*Radiation Res.* **25**, 92–102 (1965)
Albright, J. F., and Takashi Makinodan	Dynamics of expression of competence of antibody-producing cells	Pp. 427–46 in *Molecular and Cellular Basis of Antibody Formation, Proceedings of a Symposium Held in Prague on June 1–5, 1964* (ed. by J. Sterzl *et al.*), Publishing House of the Czechoslovak Academy of Sciences, Prague, 1965
Allen, R. C., R. A. Popp, and Dorothy J. Moore	Separation and relative quantitation of mouse plasma esterases with disc electrophoresis	*J. Histochem. Cytochem.* **13**, 249–54 (1965)
Anderson, N. G. (Program Coordinator)	The Joint National Institutes of Health–Atomic Energy Commission Zonal Centrifuge Development Program: semiannual report for January 1 to June 30, 1964	ORNL-3752
Arnold, W. A.	An electron-hole picture of photosynthesis	*J. Phys. Chem.* **69**, 788–91 (1965)
Barnett, W. E.	Interspecies aminoacyl-sRNA formation: fractionation of Neurospora enzymes involved in anomalous aminoacylation	*Proc. Natl. Acad. Sci. U.S.* **53**, 1462–67 (1965)
Bertsch, W. F., and J. R. Azzi	A relative maximum in the decay of long-term delayed light emission from the photosynthetic apparatus	*Biochim. Biophys. Acta* **94**, 15–26 (1965)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Bollum, F. J.	Degradation of the homopolymer complexes polydeoxyadenylate-polydeoxythymidylate, polydeoxyinosinate-polydeoxycytidylate, and polydeoxyguanylate-polydeoxycytidylate by deoxyribonuclease I	*J. Biol. Chem.* **240**, 2599–2601 (1965)
Brewen, J. G.	The induction of chromatid lesions by cytosine arabinoside in post-DNA-synthetic human leukocytes	*Cytogenetics*, **4**, 28–36 (1965)
Brockman, H. E., and Wini Goben	Mutagenicity of a monofunctional alkylating agent derivative of acridine in Neurospora	*Science* **147**, 750–51 (1965)
Cameron, I. L.	Macromolecular events leading to cell division in *Tetrahymena pyriformis* after removal and replacement of required pyrimidines	*J. Cell. Biol.* **25**, 9–18 (1965)
Cameron, I. L., and G. E. Stone	Relation between the amount of DNA per cell and the duration of DNA synthesis in three strains of *Tetrahymena pyriformis*	*Exptl. Cell Res.* **36**, 510–14 (1964)
Cammarano, Piero, Giovanni Giudice, and Bessie Lukes	Polyribosomes in regenerating liver	*Biochem. Biophys. Res. Commun.* **19**, 487–93 (1965)
Caro, L. G., and Maria Schnös	Tritium and phosphorus-32 in high-resolution autoradiography	*Science* **149**, 60–62 (1965)
Chandley, Ann C.	Application of the "distributive pairing" hypothesis to problems of segregation in translocation heterozygotes of *Drosophila melanogaster*	*Genetics* **52**, 247–58 (1965)
Chu, E. H. Y.	Effects of ultraviolet radiation on mammalian cells. I. Induction of chromosome aberrations	*Mutation Res.* **2**, 75–94 (1965)
Congdon, C. C, M. A. Kastenbaum, and D. A. Gardiner	Factors affecting mortality from secondary disease in mouse radiation chimeras	*J. Natl. Cancer Inst.* **35**, 227–34 (1965)
Cook, W. H., and R. A. Wallace	Dissociation and subunit size of β-lipovitellin	*Can. J. Biochem.* **43**, 661–70 (1965)
Cudkowicz, Gustavo, and J. H. Stimpfling	Hybrid resistance controlled by H-2 region: correction of data	*Science* **147**, 1056 (1965)
Davidson, Douglas	Cytological chimaeras in roots of *Vicia faba*	*Botan. Gaz.* **126**, 149–54 (1965)
Davis, M. L., E. B. Darden, Jr., and G. E. Cosgrove	Early hematologic effects of whole-body 14 MeV neutron irradiation in mice	*Acta Radiol.* **3**, 87–95 (1965)
Donnellan, J. E., Jr., Ella H. Nags, and H. S. Levinson	Nucleic acid synthesis during germination and postgerminative development	Pp. 152–61 in *Spores III* (ed. by L. L. Campbell and H. O. Halvorson), American Society for Microbiology, Ann Arbor, Mich., 1965
Donnellan, J. E., Jr., and R. B. Setlow	Thymine photoproducts but not thymine dimers found in ultraviolet-irradiated bacterial spores	*Science* **149**, 308–10 (1965)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Ehling, U. H.	The frequency of x-ray induced dominant mutations affecting the skeleton of mice	*Genetics* **51**, 723–32 (1965)
Foard, D. E., A. H. Haber, and T. N. Fishman	Initiation of lateral root primordia without completion of mitosis and without cytokinesis in uniseriate pericycle	*Am. J. Botany* **52**, 580–90 (1965)
Fritz, P. J., and K. B. Jacobson	Multiple molecular forms of lactate dehydrogenase	*Biochemistry* **4**, 282–89 (1965)
Gengozian, Nazareth, Barbara Rabette, and C. C Congdon	Abnormal immune mechanisms in allogeneic radiation chimeras	*Science* **149**, 645–47 (1965)
Goodman, Joan Wright	A search for parental antigens not expressed in F_1 hybrid mice	*Transplantation* **3**, 190–201 (1965)
Grell, E. H., K. B. Jacobson, and J. B. Murphy	Alcohol dehydrogenase in *Drosophila melanogaster:* isozymes and genetic variants	*Science* **149**, 80–82 (1965)
Grell, Rhoda F., and Ann C. Chandley	Evidence bearing on the coincidence of exchange and DNA replication in the oocyte of *Drosophila melanogaster*	*Proc. Natl. Acad. Sci. U.S.* **53**, 1340–46 (1965)
Hanna, M. G., Jr.	Germinal center changes and plasma cell reaction during the primary immune response	*Intern. Arch. Allergy Appl. Immunol.* **26**, 230–51 (1965)
Hanna, M. G., Jr., and C. J. Wust	Actinomycin D effect on the primary immune response in mice: a histologic and serologic study	*Lab. Invest.* **14**, 272–84 (1965)
Heddle, John A.	Lack of interaction of x-ray- and neutron-induced chromosome breaks in barley	*Mutation Res.* **2**, 149–55 (1965)
Hollaender, Alexander, S. F. Carson, and J. L. Liverman	Biology Division semiannual progress report for period ending February 15, 1965	ORNL-3768
Jacobson, K. B., and D. M. Prescott	The nucleotide pools for thymidine and cytidine in *Tetrahymena pyriformis*	*Exptl. Cell Res.* **36**, 561–67 (1964)
Jagger, John	Photochemistry of nucleic acids and its biological implications (rapporteur's report)	Pp. 59–80 in *Recent Progress in Photobiology: The Proceedings of an International Congress Held at Oxford, July 1964, Under the Auspices of the Comité International de Photobiologie* (ed. by E. J. Bowen), Blackwell Scientific Publications, Oxford, 1965
	Photoreactivation and photoprotections	*Photochem. Photobiol.* **3**, 451–61 1965)
Johnson, E. J., and H. D. Peck, Jr.	Coupling of phosphorylation and carbon dioxide fixation in extracts of *Thiobacillus thioparus*	*J. Bacteriol.* **89**, 1041–50 (1965)
Kaji, Akira, Hideko Kaji, and G. D. Novelli	Souble amino acid–incorporating system. I. Preparation of the system and nature of the reaction	*J. Biol. Chem.* **240**, 1185–91 (1965)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Kaji, Akira, Hideko Kaji, and G. D. Novelli	Soluble amino acid–incorporating system. II. Soluble nature of the system and the characterization of the radioactive product	*J. Biol. Chem.* **240**, 1192–97 (1965)
Karasaki, Shuichi	Intranuclear crystal within the phagocytes of the ovary of *Arbacia punctulata*	*J. Cell Biol.* **25**, 654–60 (1965)
Kastenbaum, M. A., and M. G. Hanna, Jr.	Statistical analysis of autoradiographic and histologic data	*Arch. Pathol.* **79**, 462–65 (1965)
Kenney, F. T., D. L. Greenman, and W. D. Wicks	RNA synthesis in response to steroid hormones	Pp. 269–75 in *Mechanisms of Hormone Action. A NATO Advanced Study Institute, Meersburg/Bodensee, 20–26 Mai 1964* (ed. by P. Karlson), Georg Thieme Verlag, Stuttgart, and Academic Press, New York and London, 1965
Khym, J. X.	Separation of transfer ribonucleic acids by a simple extraction process	*J. Biol. Chem.* **240**, PC 1488–89 (1965)
Kimball, R. F.	Studies on radiation mutagenesis in micro-organisms	Pp. 227–34 in *Genetics Today; Proceedings of the XI International Congress of Genetics, The Hague, The Netherlands, September 1963*, vol. 2 (ed. by S. J. Geerts), Pergamon, Oxford, 1965
Kimball, R. F., and Stella W. Perdue	Autoradiographic studies on the conservation of label from ribonucleosides and amino acids in *Paramecium aurelia*	*Exptl. Cell Res.* **38**, 660–69 (1965)
Kølmark, H. G.	Protection by endogenous anoxia against the mutagenic and lethal effects of x rays in *Neurospora crassa*	*Mutation Res.* **2**, 222–28 (1965)
	Protection by 2-mercaptoethylamine against the mutagenic and lethal effects of x rays in *Neurospora crassa*	*Mutation Res.* **2**, 229–35 (1965)
Kuwabara, Seishi, M. J. Cormier, L. S. Dure, Paul Kreiss, and Peter Pfuderer	Crystalline bacterial luciferase from *Photobacterium fischeri*	*Proc. Natl. Acad. Sci. U.S.* **53**, 822–28 (1965)
Long, T. J., and A. H. Haber	Growth of embryos on synthetic media after excision from gamma-irradiated (525 kr) and unirradiated wheat grains	*Radiation Botany* **5**, 223–31 (1965)
	Opposite effects of galacturonic acid on initial versus subsequent elongation of seminal roots of germinating wheat	*Botan. Gaz.* **126**, 97–100 (1965)
Mazur, Peter	Causes of injury in frozen and thawed cells	*Federation Proc.* **24**, S175–82 (1965)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
McGrath, R. A., W. M. Leach, and J. G. Carlson	Cell stages refractory to thymidine incorporation induced by x rays	*Exptl. Cell Res.* **37**, 39–44 (1965)
McGrath, R. A., R. W. Williams, and R. B. Setlow	Increased ^{3}H-thymidine incorporation into DNA of irradiated slime mould	*Intern. J. Radiation Biol.* **8**, 373–80 (1965)
Miller, O. L., Jr., Romance F. Carrier, and R. C. von Borstel	In situ and in vitro breakage of lampbrush chromosomes by x radiation	*Nature* **206**, 905–8 (1965)
Nettesheim, Paul, and Takashi Makinodan	Differentiation of lymphocytes undergoing an immune response in diffusion chambers	*J. Immunol.* **94**, 868–76 (1965)
Nishimura, Susumu, and G. D. Novelli	Dissociation of amino acid acceptor function of sRNA from its transfer function	*Proc. Natl. Acad. Sci. U.S.* **53**, 178–84 (1965)
Norton, I. L., and J. I. Valencia	Genetic extent of the deficiencies formed by combining the left end of In(1)y^4 with the right end of In(1)sc^9	*Drosophila Info. Ser.* **40**, 40 (1965)
Peck, H. D., Jr., T. E. Deacon, and J. T. Davidson	Studies on adenosine 5′-phosphosulfate reductase from *Desulfovibrio desulfuricans* and *Thiobacillus thioparus* I. The assay and purification	*Biochim. Biophys. Acta* **96**, 429–46 (1965)
Perkins, E. H., and Takashi Makinodan	The suppressive role of mouse peritoneal phagocytes in agglutinin response	*J. Immunol.* **94**, 765–77 (1965)
Popp, Diana M., and D. B. Amos	An H-2 analysis of strain RFM/U mice	*Transplantation* **3**, 501–8 (1965)
Popp, R. A.	Loci linkage of serum esterase patterns and oligosyndactyly	*J. Heredity* **56**, 107–8 (1965)
	The separation and amino acid composition of the tryptic peptides of the α chain of hemoglobin from C57BL mice	*J. Biol. Chem.* **240**, 2863–67 (1965)
Popp, R. A., and Gustavo Cudkowicz	Independence of deficient early growth and later regression of (C57BL $\times$ 101)F$_2$ marrow grafts in (C57BL $\times$ 101)F$_1$ hybrid mice	*Transplantation* **3**, 155–60 (1965)
Ritossa, F. M., J. F. Pulitzer, H. Swift, and R. C. von Borstel	On the action of ribonuclease in salivary gland cells of *Drosophila*	*Chromosoma* **16**, 144–51 (1965)
Roberts, P. A.	Difference in the behaviour of eu- and heterochromatin: crossing-over	*Nature* **205**, 725–26 (1965)
Russell, W. L.	Evidence from mice concerning the nature of the mutation process	Pp. 257–64 in *Genetics Today, Proceedings of the XI International Congress of Genetics, The Hague, The Netherlands, September 1963*, vol. 2 (ed. by S. J. Geerts), Pergamon, Oxford, 1965

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Schuel, Herbert, L. Lorand, Regina Schuel, and N. G. Anderson	Isolation of relaxing particles from rat skeletal muscles in zonal centrifuges	*J. Gen. Physiol.* **48,** 737–52 (1965)
Setlow, Jane K.	Effects of uv on DNA: correlations among biological changes, physical changes, and repair mechanisms	*Photochem. Photobiol.* **3** 405–13 (1965)
Setlow, Jane K., M. E. Boling, and F. J. Bollum	The chemical nature of photoreactivable lesions in DNA	*Proc. Natl. Acad. Sci. U.S.* **53,** 1430–36 (1965)
Setlow, R. B., W. L. Carrier, and F. J. Bollum	Pyrimidine dimers in uv-irradiated poly dI:dC	*Proc. Natl. Acad. Sci. U.S.* **53,** 1111–18 (1965)
Seto, Frank, and J. F. Albright	An analysis of host and donor contributions to splenic enlargement in chick embryos inoculated with adult chicken spleen cells	*Develop. Biol.* **11,** 1–24 (1965)
Shepard, D. C.	Production and elimination of excess DNA in ultraviolet-irradiated Tetrahymena	*Exptl. Cell Res.* **38,** 570–79 (1965)
Smith, L. H., and Phan The Tran	Long-term preservation of bone marrow	*Nature* **205,** 503–4 (1965)
Stevens, Barbara J.	The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell	*J. Cell Biol.* **24,** 349–68 (1965)
Stone, G. E., and O. L. Miller, Jr.	A stable mitochondrial DNA in *Tetrahymena pyriformis*	*J. Exptl. Zool* **159,** 33–37 (1965)
Stone, G. E., O. L. Miller, Jr., and D. M. Prescott	H^3-Thymidine derivative pools in relation to macronuclear DNA synthesis in *Tetrahymena pyriformis*	*J. Cell Biol.* **25,** 171–77 (1965)
Swartzendruber, D. C., and M. G. Hanna, Jr.	Electron microscopic autoradiography of germinal center cells in mouse spleen	*J. Cell Biol.* **25,** 109–119 (1965)
Takata, Chinami, J. F. Albright, and Tuneo Yamada	Lens fiber differentiation and gamma crystallins: immunofluorescent study of Wolffian regeneration	*Science* **147,** 1299–1301 (1965)
Trosko, J. E., E. H. Y. Chu, and W. L. Carrier	The induction of thymine dimers in ultraviolet-irradiated mammalian cells	*Radiation Res.* **24,** 667–72 (1965)
Trosko, J. E., and Sheldon Wolff	Strandedness of *Vicia faba* chromosomes as revealed by enzyme digestion studies	*J. Cell Biol.* **26,** 125–35 (1965)
Tyndall, R. L., J. G. Vidrine, Ernestine Teeter, A. C. Upton, W. W. Harris, and M. A. Fink	Cytopathogenic effects in a cell culture infected with a murine leukemia virus	*Proc. Soc. Exptl. Biol. Med.* **119,** 186–89 (1965)
Von Halle, Elizabeth S.	Localization of *E-spl*	*Drosophila Info. Serv.* **40,** 60 (1965)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Walburg, H. E., Jr., Edna I. Mynatt, G. E. Cosgrove, R. L. Tyndall, and D. M. Robie	Microbiological evaluation of an isolation facility for the production of specific-pathogen-free mice	*Lab. Animal Care* **15**, 208–216 (1965)
Wallace, R. A.	Resolution and isolation of avian and amphibian yolk-granule proteins using TEAE-cellulose	*Anal. Biochem.* **11**, 297–311 (1965)
Webber, B. B., and F. J. de Serres	Induction kinetics and genetic analysis of x-ray-induced mutations in the *ad-3* region of *Neurospora crassa*	*Proc. Natl. Acad. Sci. U.S.* **53**, 430–37 (1965)
Whitson, G. L., and G. M. Padilla	The effects of actinomycin D on stomatogenesis and cell division in temperature-synchronized *Tetrahymena pyriformis*	*Exptl. Cell Res.* **36**, 667–71 (1964)
Willard, Henrianne G., I. B. H. Hoppe, and Paul Nettesheim	Mouse leukocytes in culture	*Proc. Soc. Exptl. Biol. Med.* **118**, 993–96 (1965)
Wolff, Sheldon	Are sister chromatid exchanges sister strand cross-overs or radiation-induced exchanges?	*Mutation Res.* **1**, 337–43 (1964)
	Radiation effects as measured by chromosome damage	Pp. 167–79 in *Cellular Radiation Biology, A Collection of Papers Presented at the 18th Annual Symposium on Fundamental Cancer Research, 1964*, published for the University of Texas M. D. Anderson Hospital and Tumor Institute by Williams and Wilkins, Baltimore, Md., 1965
Wust, C. J., and M. G. Hanna, Jr.	Time relationship of injection of actinomycin D and antigen to the immune response	*Proc. Soc. Exptl. Biol. Med.* **118**, 1027–31 (1965)
(Members of the Biology Division)	Abstracts presented at the Bone Marrow Transplantation and Chemical Radiation Protection Discussion Meeting Held April 10, 1965, Atlantic City, N.J. (organized by L. J. Cole and C. C Congdon)	*Experimental Hematology*, No. 8 (1965)

LECTURES

During the period February 16, 1965, to July 31, 1965, members of the Biology Division presented 247 lectures and speeches. This figure compares with 219 for the first report period of 1964 and with 242 for the August 16, 1964–February 15, 1965, reporting period. Of the 247 lectures and speeches, a total of 65 was given at meetings of professional societies, and 170 on invitation of universities, laboratories, and scientific institutions in the United States and abroad. A total of 12 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 cell division and radiation sensitivity	University of Florida, Gainsville
Adler, H. I. (and Alice A Hardigree)	The growth and division of irradiated bacteria [*Bacteriol. Proc.* 39 (1965)]	Am. Soc. Microbiol. Atlantic City, N.J.
Albright, J. F.	Competence of cells for antibody formation	Marine Biological Laboratory, Woods Hole, Mass.
	Cytokinetic events in soluble antibody production	Duke University School of Medicine, Durham, N.C.
Allen, R. C. (and Dorothy J. Moore)	A discontinuous vertical flat bed electrophoresis system for the study of plasma and tissue esterases in acrylamide gel	Histochemistry Society, Philadelphia, Pa.
Anderson, N. G.	1. Introduction to zonal centrifugation 2. S-ρ techniques 3. Continuous-flow rotors 4. New experimental rotor systems	Training Conference and Symposium on Zonal Centrifuge Systems, ORNL and K-25 Powerhouse, Oak Ridge, Tenn.
	The application and theory of zonal centrifuge	Squibb Institute for Med. Research, New Brunswick, N.J.
	Review of the proposed MAN program	ORINS Annual Conference, Oak Ridge, Tenn.
	Research on the liquid centrifuge	U.S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Arnold, W. A.	Delayed light and photosynthesis	1. Johnson Research Foundation, Philadelphia, Pa. 2. Oregon State University, Corvallis
	Problems concerning the mechanism of photosynthesis	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
Barber, A. A.	Isolation of native glycogen by combined rate and isopycnic centrifugation	Training Conference and Symposium on Zonal Centrifuge Systems, ORNL and K-25 Powerhouse, Oak Ridge, Tenn.
Barnett, W. E.	Coding properties of sRNA's	1. Vanderbilt University, Nashville, Tenn. 2. University of Texas, Austin
Bender, M. A	Cytogenetic effects of leprechaunism	American Neurological Association, Atlantic City, N.J.
	The use of chromosome aberration analysis for radiation dosimetry	New York University, Sterling Forest
Bennett, Michael (and G. M. Shearer)	Stem cells and lymphocytes in mouse bone marrow [*Federation Proc.* **24**, 240 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Soc. Exptl. Pathology), Atlantic City, N.J.
Bollum, F. J.	Biosynthetic polydeoxynucleotides	Los Alamos Scientific Laboratory, Los Alamos, N.M.
	Polydeoxycytidylate and random copolymers of deoxynucleotides [*Federation Proc.* **24**, 348 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Brewen, J. G.	The relation between the cell cycle and x-ray-induced chromosome aberrations	University of Rochester, Rochester, N.Y.
	The relation between x-ray-induced aberration frequencies and the mitotic cycle in the chromosomes of human leukocytes [*Radiation Res.* **25**, 177 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
Cameron, I. L. (G. M. Padilla and Betty M. Wysinger)	Regulation of the DNA-synthetic period within the cell cycle of *Tetrahymena pyriformis*	2d International Conference of Protozoology, London
Caro, L. G.	Mechanism of DNA transfer in bacterial mating	California Institute of Technology, Pasadena
Carson, S. F.	Modern experimental biology: the synthesis of mathematics, physical science, and biology	13th Annual Southern Appalachian Science Fair Awards Dinner, Knoxville, Tenn.
	Biotin and B_{12} coenzyme catalyzed reactions in the interconversion of propionate and succinate: 1. Propionyl carboxylase and the transcarboxylases 2. Methylmalonyl isomerase and recemase	University of Georgia, Athens
	Modern experimental biology: an exercise in little and big science in a national laboratory	ORINS Annual Conference, Oak Ridge, Tenn.
Chu, E. H. Y.	1. Cytogenetic effects of radiation in mammalian cells grown in culture 2. Human cytogenetics	Converse College, Spartanburg, S.C.
	1. The mammalian X chromosome 2. The effect of ultraviolet light on mammalian chromosomes	Florida State University, Tallahassee
	Radiation sensitivity in synchronized mammalian cells	Ohio Academy of Sciences Annual Meeting, Ohio University, Athens
	The effect of ultraviolet light on mammalian chromosomes	University of Wisconsin, Madison
Clapp, N. K.	Histopathologic changes in the kidney of the chick embryo following x irradiation [*Radiation Res.* **25**, 180 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
	Mechanisms involved in experimental tumor infection in mice following administration of nitrosamines	Colorado State University, Fort Collins
	1. Circulatory disturbances (two lectures) 2. Growth and development	Survey of General Pathology, University of Tennessee Summer Course, Oak Ridge, Tenn.
Cline, G. B.	Continuous-flow centrifugation with banding	Training Conference and Symposium on Zonal Centrifuge Systems, ORNL and K-25 Powerhouse, Oak Ridge, Tenn.
Cohn, W. E.	Some aspects of the chemistry and biochemistry of pseudouridine	1. University of Kansas, Lawrence 2. University of Nebraska, Lincoln 3. University of Colorado, Denver

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Congdon, C. C	Lymph node weight in radiation chimeras [*Federation Proc.* **24**, 636 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
	ORGAN transplantation	1. Knoxville Surgical Society, Knoxville, Tenn. 2. ORINS Annual Conference, Oak Ridge, Tenn.
Congdon, C. C (M. A. Kastenbaum and D. A. Gardiner)	Reduced mortality from secondary disease in radiation chimeras	Bone Marrow Conference, Atlantic City, N.J.
Cosgrove, G. E.	Pathology of parasitism	Wayne State University, Detroit, Mich.
	Diseases in a primate colony	ORINS Staff Meeting, Oak Ridge, Tenn.
	Biological effects of radiation	ORNL Radiation Safety Training Program, Oak Ridge, Tenn.
	Delayed effects of radiation	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
	Radiobiological studies on snakes and box turtles	Symposium of Delayed Effects Group, Radiation Research Society, Philadelphia, Pa.
	Delayed effects of radiation in mammals	ORSORT, Oak Ridge, Tenn.
	Infection: bacterial, viral, parasitic (two lectures)	Survey of General Pathology, University of Tennessee Summer Course, Oak Ridge, Tenn.
Cudkowicz, Gustavo	The immunogenetic basis of hybrid resistance to parental marrow grafts	International Symposium on Isoantigens and Cell Reactions, Wistar Institute, Philadelphia, Pa.
	Sex-associated hybrid resistance to parental marrow grafts [*Federation Proc.* **24**, 637 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Soc. Exptl. Pathology), Atlantic City, N.J.
	Genetics of marrow transplantation in mice	Roswell Park Memorial Institute, Buffalo, N.Y.
	Immunogenetics of hybrid resistance to parental marrow grafts	The Jackson Laboratory, Bar Harbor, Me.
Curtiss, Roy, III	Genetic transfer by conjugation in *Escherichia coli*	1. University of Delaware, Newark 2. Tuskegee Institute, Tuskegee, Ala.
	UV-induced genetic recombination in a partial diploid strain of *Escherichia coli*	University of Delaware, Newark
	Bacterial lysogeny (two lectures)	Bacterial Genetics Course, University of Tennessee, Knoxville
	Mechanism of genetic recombination in bacteria	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
Curtiss, Roy, III (Claire M. Berg and L. J. Charamella)	Mutant isolation by a D-cycloserine enrichment cycling technique [*Bacteriol. Proc.* 17 (1965)]	Am. Soc. Microbiol., Atlantic City, N.J.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Darden, E. B., Jr.	Effect of radiation on biological organisms	U.S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Darden, E. B., Jr. (A. C. Upton, M. L. Randolph, G. E. Cosgrove, K. W. Christenberry, and J. W. Conklin)	Life shortening, neoplasia, and cataractogenesis in mice irradiated with 14-Mev fast neutrons [*Radiation Res.* **25**, 183 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
Dolin, M. I.	NADH peroxidase	Symposium on Flavins and Flavoproteins, Amsterdam, The Netherlands
Dolin, M. I. (and R. H. Baum)	Localization of electron transport components in *Streptococcus faecalis*, 10C1 [*Bacteriol. Proc.* 96–97 (1965)]	Am. Soc. Microbiol., Atlantic City. N.J.
Epler, J. L.	1. sRNA's and coding in Neurospora 2. EMS-induced mosaics in Drosophila	Illinois State University, Normal
Epler, J. L. (W. E. Barnett and K. B. Jacobson)	Coding properties of anomalously charged sRNA's [*Federation Proc.* **24**, 408 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
Finamore, F. J.	Recent advances in the biochemistry of development	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
Fisher, W. D.	Research in biophysics, isolation of subcellular particles	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
	B-X rotor system	Training Conference and Symposium on Zonal Centrifuge Systems, ORNL and K-25 Powerhouse, Oak Ridge, Tenn.
	Science and society	Roxboro High School, Roxboro, N.C.
Foard, D. E.	The cell and the organism	Spring Hill College, Mobile, Ala.
Goodman, Joan Wright	Failure of parental tissues to immunize F_1 hybrids [*Federation Proc.* **24**, 180 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
Green, J. G. (and N. G. Anderson)	Prototype automatic carbohydrate analyzer [*Federation Proc.* **24**, 606 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Physiol. Soc.), Atlantic City, N.J.
Gude, W. D.	1. Biological effects of radiation in mammals 2. Autoradiography as a tool in research	Lanier High School, Maryville, Tenn.
	1. Pathology defined – nature of disease 2. Degeneration	Survey of General Pathology, University of Tennessee Summer Course, Oak Ridge, Tenn.
	Basic radiobiology	U.S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Hanna, M. G., Jr.	The role of lymphatic germinal centers in the immune response	International Conference of Radiation and Immune Response, London
	Lymphatic tissue changes during the primary immune response	University of Bern, Bern, Switzerland

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Hellman, Alfred	The influence of hormones on viral infection	National Cancer Institute Information Meeting, Oak Ridge, Tenn.
Hellman, Alfred (and D. J. Giron)	Rapid isolation and identification of a prototype viral agent [*Bacteriol. Proc.* 111–12 (1965)]	Am. Soc. Microbiol., Atlantic City, N.J.
Hollaender, Alexander	The future of radiation biology	Brookhaven National Laboratory, Upton, N.Y.
	Studies in radiation at the Oak Ridge National Laboratory	Marine Biological Laboratory, Woods Hole, Mass.
	Modern developments in radiation biology	Southeastern Obstetrical and Gynecological Society, Oak Ridge, Tenn.
	Studies going on in Biology Division, Oak Ridge National Laboratory	1. Institute of Radiation Biology and Radiation Hygiene, Budapest, Hungary 2. Institute of Genetics, Budapest, Hungary 3. University of Pécs, Institute of Biophysics, Pécs, Hungary 4. Biological Research Institute, Hungarian Academy of Science, Lake Balaton, Tihany, Hungary 5. Radiobiological Laboratory, Institute of Nuclear Science, "Boris Kidrich," Belgrade, Hungary 6. Institute of Nuclear Sciences, "Ruder Bošković," Zagreb, Yugoslavia 7. Nuclear Institute "Jožef Stefan," Ljubljana, Yugoslavia
	Research Problems in Biology Division, Oak Ridge National Laboratory	Scientists Club, Hungarian Academy of Science, Budapest
Jacobson, K. B.	Anomalous aminoacyl-sRNA formation by heterologous enzymes	University of Illinois, Urbana
	1. Anomalies in formation of aminoacyl sRNA 2. Isozymes of alcohol dehydrogenase in Drosophila	Auburn University, Auburn, Ala.
Jenkins, V. K. (A. C. Upton and T. T. Odell, Jr.)	Viral induction of myeloid leukemia: inhibition by estrogen [*Cancer Res.* **6**, 33 (1965)] (not presented orally)	Am. Assoc. for Cancer Research, 56th Annual Meeting, Philadelphia, Pa.
Kelmers, A. D.	The presentation of transfer RNA by reverse phase chromatography [*Federation Proc* **24**, 482 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
Kenney, F. T.	Hydrocortisone stimulation of RNA synthesis in induction of hepatic enzymes	Symposium on Hormonal Control of Protein Biosynthesis, Gatlinburg, Tenn. (ORNL Biology Division Symposium)

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Kenney, F. T.	Steroid hormones in the control of genetic activity	State University of New York, Plattsburgh
Kimball, R. F.	1. Repair of premutational damage in *Paramecium* 2. Repair from premutation damage	Western Researve University, Cleveland, Ohio
	Comparison between the mutation process with triethylenemelamine and with x rays in *Paramecium aurelia* [*Radiation Res.* **25**, 206 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
	The cellular effects of radiation	ORINS Annual Conference, Oak Ridge, Tenn.
Lion, M. B.	UV photoproducts in 5-bromouracil substituted DNA of *Escherichia coli* 15 T^{-1} [*Radiation Res.* **25**, 211 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
Makinodan, Takashi	Growth and senescence of the antibody-forming potential of the spleen	St. Jude Research Hospital, Memphis, Tenn.
	Cellular dynamics of immune response	Symposium on Immunological Tolerance, Lake Locarno, Switzerland
	The effect of radiation on the immune condition	Conference on Radiation and Immune Response, Chester Beatty Research Institute, London
	Growth and senescence of the immune mechanism	1. Institute of Nuclear Sciences "Boris Kidrich," Belgrade, Yugoslavia 2. Institute of Nuclear Sciences, "Rudjer Bošković," Zagreb, Yugoslavia
Malling, H. V.	Mutagenic action of nitrous acid in *Neurospora* and the mutagenic action of antineoplastic compounds	Danish Microbial Society, Copenhagen
Manney, T. R.	Yeast tryptophan synthetase	University of Cincinnati, Cincinnati, Ohio
	Gene control of enzyme formation	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
Mattingly, M. Ellen	Studies on the cell cycle and differentiation using 5-aminouracil and other chemicals	University of Tennessee, Knoxville
	Recent advances in the biological uses of radioactivity	LeMoyne College, Memphis, Tenn.
Mazur, Peter	The role of water, permeability, and temperature in living cells	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
McGrath, R. A.	Repair of irradiated DNA	1. Tuskegee Institute, Tuskegee, Ala. 2. North Carolina State University, Raleigh 3. Ohio State University, Columbus
Mead, C. G. (and Elliot Volkin)	The oligodeoxyribonucleotide transferase system [*Federation Proc.* **24**, 349 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Miller, O. L., Jr.	Fine structure of lampbrush chromosomes	1. Yale University, New Haven, Conn. 2. University of Minnesota Medical School, Minneapolis
	Chromosome structure	1. Florida State University, Tallahassee 2. Vanderbilt University, Nashville, Tenn.
	Electron microscopy of chromosomes	Winthrop College, Rock Hill, S.C.
	1. Chromosome structure and function 2. Nucleolar structure and function 3. Structure and metabolism of the nuclear-cytoplasmic boundary	University of Minnesota, St. Paul
Nettesheim, Paul (and Takashi Makinodan)	Recovery of secondary antibody producing potential of mouse spleen cells after x irradiation [*Federation Proc.* **24**, 377 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Assn. Immunologists), Atlantic City, N.J.
Novelli, G. D.	Protein synthesis by regenerating liver	1. University of Georgia, Athens 2. Brandeis University, Waltham, Mass. 3. Oregon State University, Corvallis
Novelli, G. D. (Piero Cammarano, Marialuisa Melli, and Giovanni Giudice)	Polyribosome formation and amino acid incorporation during liver regeneration [*Federation Proc.* **24**, 484 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
Oakberg, E. F.	Radiation damage to the gonads	University of Tennessee Institute of Radiation Biology, Knoxville
	Genetic effects of radiation	U.S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Odell, T. T., Jr.	Regulation of blood platelets (three lectures)	Western Kentucky State College, Bowling Green
	Biological effects of radiation on mammals	U.S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Odell, T. T., Jr. (T. P. McDonald and C. W. Jackson)	Size distribution of blood platelets after x radiation [*Radiation Res.* **25**, 223 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
Padilla, G. M. (and A. A. Barber)	Studies of native glycogen isolated from synchronized *Tetrahymena pyriformis* (read by title only)	International Conference on Protozoology, London
Perkins, E. H.	Effect of irradiation on intracellular digestion by peritoneal leukocytes	Commission on Radiation and Infection, Armed Forces Epidemilogical Board, Washington, D.C.
Perkins, E. H. (and Toshiteru Morita)	Effect of x irradiation on the phagocytosis of foreign erythrocytes [*Federation Proc.* **24**, 381 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Assn. Immunologists), Atlantic City, N.J.
Popp, R. A.	Hemoglobin variants in mice	Fed. Am. Soc. Exptl. Biol. (Am. Soc. Exptl. Pathology), Atlantic City, N.J.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Popp, R. A. (C. C Congdon and Joan Wright Goodman)	Spleen weight as a measure of bone marrow cell growth: comparisons with spleen colony formation and ^{59}Fe uptake	Fed. Am. Soc. Exptl. Biol. (Bone Marrow Conference), Atlantic City, N.J.
Regan, J. D.	Patterns of nucleic acid synthesis in human cell cultures	1. Florida State University, Tallahassee 2. University of Miami, Miami, Fla.
Regan, J. D. (and E. H. Y. Chu)	A convenient method for periodic assay of DNA synthesis in synchronous human cells in suspension culture	Tissue Culture Association, 16th Annual Meeting, Miami Beach, Fla.
Rogers, Stanfield	Concerning the helicity of Shope virus induced arginase and its significance [*Federation Proc.* **24**, 309 (1965)]	Am. Soc. Exptl. Biol. (Am. Soc. Exptl. Pathology), Atlantic City, N.J.
Russell, Liane B.	Death and chromosome damage from irradiation of preimplantation stages	CIBA Symposium on Preimplantation Stages of Pregnancy, London
	Genetic effects of radiation	ORSORT, Oak Ridge, Tenn.
Russell, W. L.	Recent results on the genetic effects of radiation in mice	ORNL Health Physics Division, Oak Ridge, Tenn.
	The scientific method	Tennessee Junior Science Day, Oak Ridge, Tenn. (Tennessee Academy of Science)
	Studies in mammalian radiation genetics	University of Tennessee, Knoxville
	LET effects in radiation physics, chemistry and Genetics: mammals	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
	New findings affecting the estimation of genetic hazards of radiation	Health Physics Society, Los Angeles
	Radiation and mammalian heredity	ORINS Annual Conference, Oak Ridge, Tenn.
Serrano, L. J.	Animals in research	Georgia Women's College, Milledgeville
	Radiation biology: preparation for an experiment with low level irradiation	U.S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Setlow, Jane K.	Chemical nature of photoreactivable lesions in DNA	California Institute of Technology, Pasadena
Setlow, R. B.	The effects of ultraviolet light on polynucleotides: physical, chemical and biological effects	M. D. Anderson Hospital, The University of Texas, Houston
	A molecular error-correcting mechanism in DNA	1. Reed College, Portland, Ore. 2. University of British Columbia, Vancouver, British Columbia, Canada
	Effects of uv on polynucleotides in vitro and in vivo	Oregon State University, Corvallis
	Effects of uv on polynucleotides	Harvard Medical School, Boston, Mass.
Shearer, G. M.	Single hemagglutinin-releasing cells (read by title) [*Federation Proc.* **24**, 305 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Soc. for Exptl. Pathology), Atlantic City, N.J.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Sheeler, Phillip	Iron incorporation by rabbit and turtle reticulocytes	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
Smith, L. H.	Effect of temperature on radiation damage to mouse bone marrow cells in culture [*Radiation Res.* **25**, 241–42 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
Stapleton, G. E.	Effects of radiation on microorganisms	1. Centenary College of Louisiana, Shreveport 2. Louisiana Polytechnic Institute, Ruston 3. Northwestern State College of Louisiana, Natchitoches
	Development of radioresistance in cultures of a purineless mutant of *Escherichia coli* K-12 [*Radiation Res.* **25**, 244 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
Stapleton, G. E. (and W. D. Fisher)	Ribosomal profiles of radiation sensitive and resistant *Escherichia coli* [*Bacteriol. Proc.* 87–88 (1965)]	Am. Soc. Microbiol., Atlantic City, N.J.
Stulberg, M. P.	Interactions of amino acids activating enzymes and transfer RNA	1. Virginia Polytechnic Institute, Blacksburg 2. University of Maryland, College Park 3. Auburn University, Auburn, Ala. 4. Vanderbilt University, Nashville, Tenn.
Swartzendruber, D. C.	Electron microscopic observations on splenic white pulp during the early intervals of a primary immune response [*Anat. Record* **15**, 422–23 (1965)]	Am. Assoc. Anatomists, 78th Ann. Meeting, Miami Beach, Fla.
	Electron microscopic studies on lymphatic tissue	Seton Hall University, South Orange, N.J.
Tyndall, R. L.	Viruses, a continuing challenge (three lectures)	Western Maryland College, Westminster
Tyndall, R. L. (K. B. Jacobson and Ernestine Teeter)	Fingerprint analysis of viral and cellular RNA [*Bacteriol. Proc.* 106–7 (1965)]	Am. Soc. Microbiol., Atlantic City, N.J.
Upton, A. C.	Radiation and cancer	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
	The importance of *cum laude* in science and society	Webb School, Knoxville, Tenn.
	1. The scientist and the crisis of tomorrow 2. Radiation carcinogenesis 3. Relative biological effectiveness of radiation of different types: review of the subject	Oregon State University, Corvallis
	Radiation carcinogenesis: implications for biology, medicine, and public health	University of Washington, Seattle

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Upton, A. C.	Radiobiological observations on radiation cancer	Radiobiological Association, Washington, D.C.
	Radiation hazards to man	1. MSRE Operation, ORNL, Oak Ridge, Tenn. 2. Reactor Division, ORNL, Oak Ridge, Tenn.
	Disease and aging in irradiated individuals	ORINS Annual Conference, Oak Ridge, Tenn.
	1. The pathology of aging (two lectures) 2. The pathology of radiation (two lectures)	Survey of General Pathology, University of Tennessee Summer Course, Oak Ridge, Tenn.
Uziel, Mayo (and W. E. Cohn)	Column desalting procedures in the recovery of oligonucleotides from chromatographic effluents [*Federation Proc.* **24**, 668 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
Volkin, Elliot	An oligodeoxyribonucleotide transferase system	1. Albert Einstein School of Medicine, Yeshiva University, New York 2. University of Pennsylvania School of Medicine, Philadelphia, Pa. 3. Indiana University, Bloomington
von Borstel, R. C.	Suicide experiments with phosphorus^{33} in phage T4	1. University of Washington, Seattle 2. Stanford University, Stanford, Calif. 3. University of Chicago, Chicago, Ill.
	Developmental genetics, gene control during growth and differentiation	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
	Space biology	ORINS Annual Conference, Oak Ridge, Tenn.
Walburg, H. E., Jr. (Edna I. Mynatt and D. M. Robie)	Influence of strain and diet on the mortality of x-irradiated germfree and conventional mice as measured by the $LD_{50/30}$ [*Radiation Res.* **25**, 249–50 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
Wallace, R. A.	Structure and formation of vertebrate yolk granules	Carnegie Institute of Washington, Baltimore, Md.
	Biochemical aspects of vertebrate yolk formation and structure	Marine Biological Laboratory, Embryology Course, Woods Hole, Mass.
Warner, A. H.	Metabolism of diguanosine nucleotides during brine shrimp embryogenesis	University of Windsor, Windsor, Ontario, Canada
	Diguanosine nucleotides in brine shrimp eggs	University of Georgia, Athens
Warner, A. H. (and F. J. Finamore)	Occurrence of P^1, P^3-diguanosine 5′-triphosphate in brine shrimp eggs [*Federation Proc.* **24**, 669 (1965)]	Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Welshons, W. J.	The fine structure of a lethal locus in Drosophila: a cytogenetic analysis	University of North Carolina School of Medicine, Chapel Hill
	An analysis of a gene in Drosophila	Iowa State University, Ames

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Whiting, P. W.	Complexity of the *R* region in *Mormoniella* [*ASB Bull.* **12**, 54 (1965)]	Assoc. of Southeastern Biologists, University of Virginia, Charlottesville
Whitson, G. L.	1. Problems in cell division synchrony 2. Morphogenesis in ciliated protozoa	Modern Zoology Course, University of Tennessee, Oak Ridge, Tenn.
Wicks, W. D. (and F. T. Kenney)	Stimulation of transfer RNA synthetic by steroid hormones [*Federation Proc.* **24**, 600 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Willard, Henrianne G. (and L. H. Smith)	Quantitative rejection of homografted marrow by isologous tissues [*Federation Proc.* **24**, 161 (1965)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N.J.
Wolff, Sheldon	On the chemistry of chromosome continuity	University of Minnesota, Minneapolis
	Radiation genetics	St. Mary's College, Winona, Minn.
	On the origin of sister strand exchanges in tritium-labeled chromosomes [*Radiation Res.* **25**, 254 (1965)]	Radiation Research Society, 13th Annual Meeting, Philadelphia, Pa.
	Chromosome aberrations: on the nature of the site of radiation-induced nuclear damage	Gordon Research Conference, Meriden, N.H.
Wust, C. J.	Aspects of the induction phase of antibody synthesis	University of Georgia, Athens
Wust, C. J. (J. B. Murphy and S. F. Carson)	Lactic acid dehydrogenase activity as an indicator of rat erythrocyte antigen during the immune response [*Federation Proc.* **24**, 176 (1965)]	Fed. Am. Soc. Exptl. Biol. (Am. Soc. Biol. Chem.), Atlantic City, N.J.
Yamada, Tuneo	Behavior of lens specific antigens during lens regeneration	University of Göteborg, Göteborg, Sweden
	Cellular and subcellular events occurring during lens regeneration	University of Zurich, Zurich, Switzerland
Zimmerman, B. K.	The enzymic synthesis and degradation of DNA in *Micrococcus lysodeikticus*	Stanford University, Stanford, Calif.

VISITING LECTURERS

During the period February 16–July 31, 48 lectures were given on the Biology Division Seminar Program by guest speakers from scientific institutions and universities in this country and abroad. Visiting lecturers included scientists from Australia, Brazil, England, Italy, The Netherlands, Scotland, and Sweden.

SPEAKER	AFFILIATION	SUBJECT
Gerald E. Adams	Research Unit in Radiobiology, Mt. Vernon Hospital, Middlesex, England	Pulsed methods in radiation chemistry: their relevance to radiobiology
Peter Alexander	Chester Beatty Research Institute, London, England	Cancer immunology

SPEAKER	AFFILIATION	SUBJECT
LeRoy Baker	Department of Biochemistry, Michigan State University, East Lansing	Polyphosphate in synchronous cultures of Chlorella
Per-Erik Bergner	Karolinska Institute, Stockholm, Sweden; now associated with Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn.	Principles and problems of tracer dynamics (series of four lectures)
L. R. Caldas	Instituto de Biofisica, Universidade de Brasil, Rio de Janeiro, Brazil	Studies of the lysogenic system of *Staphylococcus albus*
John Cebra	Department of Microbiology, University of Florida, Gainesville	Cellular and molecular studies concerning rabbit immunoglobulin
Satya Dubey	Procter and Gamble, Cincinnati, Ohio	Some results and applications of the Weibull distribution
W. M. Elsasser	Princeton University, Princeton, N.J.	A physicist looks at non-mechanistic biology (series of four lectures)
Charles E. Gates	University of Minnesota, St. Paul	Simulation of genetic models by digital computers
Robert B. Helling	Karolinska Institute, Stockholm, Sweden	Recombination and repression in *Escherichia coli*
Bruce Holloway	University of Melbourne, School of Microbiology, Victoria, Australia	Control of host induced modifications at the chromosomal and extra chromosomal level in *Pseudomonas aeruginosa*
Ray W. Holton	University of Tennessee, Knoxville	Cytochromes in algae
Gordon Hunt	University of Tennessee, Knoxville	Amino acid metabolism in the jack bean
Albert Ketler	Public Health Research Institute of the City of New York	Some aspects of viral oncology
Robert E. Krisch	Department of Physics, University of Pennsylvania, Philadelphia	Effects of P^{32} decay in the Hfr donor on genetic recombination of *E. coli* K-12
Yvonne T. Lanni	Department of Microbiology, Emory University, Atlanta, Ga.	Molecular aspects of invasion by Phage T5
Hans Laufer	Johns Hopkins University, Baltimore, Md.	Nuclear-cytoplasmic interaction during salivary gland development
William Leach	University of Tennessee, Knoxville	Retention of H^3 thymidine in grasshopper neuroblasts
Anne McLaren	Institute of Animal Genetics, Edinburgh, Scotland	Duplex formation in mammalian DNA *in vitro*
Charles J. Mode	Montana State College, Bozeman	Stochastic calculus and its applications in genetics
M. L. Morse	University of Colorado Medical Center, Denver	Genetic and biochemical studies of carbohydrate transport in *Staphylococcus aureus*
C. W. Moss	North Carolina State University, Raleigh	Metabolic injury in frozen-thawed *E. coli*
S. Nakai	Donner Laboratory, University of California, Berkeley	Chromosomal mechanism of radiation-induced recombination in yeast
Walter E. Nance	Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tenn.	Genetic and biochemical studies of human haptoglobin

SPEAKER	AFFILIATION	SUBJECT
Agnar Nilsson	Research Institute of National Defence, Sundbyberg, Sweden	Recent observations on the effects of Sr-90-induced neoplasia
Per Oftedal	Department of Biology, City of Hope Medical Research Institute, Duarte, Calif.	Spermatogonial radiosensitivity and dose-rate effects in Drosophila
Robert Perry	Institute for Cancer Research, Philadelphia	The nucleolus and the synthesis of ribosomes
Peter M. Ray	Department of Botany, University of Michigan, Ann Arbor	The mechanism of cell wall expansion in growth of plant cells
F. M. Ritossa	Department of Microbiology, University of Illinois, Urbana	Localization of the DNA complementary to ribosomal RNA in Drosophila
Walter Sauerbier	McArdle Laboratory for Cancer Research, University of Wisconsin, Madison	Host modification of bacteriophages
G. T. Scarascia	Comitato Nazionale per l'Energia Nucleare, Casaccia, Italy	Radiogenetic studies at Casaccia, Italy
John C. Schooley	Donner Laboratory, University of California, Berkeley	Studies on the mechanism of action of erythropoietin
Jose Souto	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tenn.	Studies on chemical changes in radiation injury of mammals
Walther Stoeckenius	The Rockefeller Institute, New York	Some current problems in membrane structure research
T. T. Stonier	Manhattan College, Bronx, N.Y.	Studies on plant tumors as a theoretical model for the cancer problem
T. Sugiyama	University of California, Berkeley	Structural studies on RNA from bacteriophage MS2
A. D. Tates	United Nations, United Nations, N.Y.	Normal spermatogenesis in Drosophila as studied by electron microscopy
Kenneth V. Thimann	Harvard University, Cambridge, Mass.	Recent studies on geotropism and phototropism
D. W. van Bekkum	Radiobiological Institute, Rijswijk, The Netherlands	Evidence of secondary disease in germfree heterologous radiation chimeras
Walter S. Vincent	Department of Anatomy and Cell Biology, University of Pittsburgh, Pittsburgh, Pa.	Studies on RNA and ribosomal metabolism in stationary phase and refed yeast
John H. L. Watson	Ford Hospital, Detroit, Mich.	Ultrastructure of the bronchial mucosa. Comparison of normal, bronchitic, and bronchiogenic carcinoma epithelial cells in man
Judith Wegman	Department of Microbiology, Western Reserve University Medical School, Cleveland	Comparative studies of the tryptophan pathways in *Neurospora* and yeast

SPEAKERS AT PROFESSIONAL MEETINGS, FALL–WINTER 1965

Radiation Biology Conference, Oak Ridge, Tenn. – Alexander Hollaender, A. C. Upton, J. L. Liverman, A. H. Haber, and H. I. Adler

Second Annual Meeting of the Society for Cryobiology, Madison, Wis. – Peter Mazur (coauthor, Janice J. Schmidt) and S. P. Leibo (coauthor, Peter Mazur)

Research Conference on Instrumentation Science, Geneva, N.Y. – R. C. Allen

Pathobiology Conference, Aspen, Colo. – F. J. de Serres

Twenty-Third Annual Meeting of the Electron Microscopy Society of America, New York – R. F. Zeigel (coauthor, R. L. Tyndall)

Sixteenth Annual Meeting of the American Institute of Biological Sciences, Urbana, Ill. – Tuneo Yamada and J. L. Liverman

Mendelian Centennial Meeting of the Genetics Society of America, Fort Collins, Colo. – P. A. Roberts, U. H. Ehling, Liane B. Russell (coauthor, E. H. Y. Chu), R. F. Kimball, E. F. Oakberg, W. L. Russel (coauthor, Elizabeth M. Kelly), J. E. Trosko (coauthor, Sheldon Wolff), Liane B. Russell (coauthor, Clyde S. Montgomery), Sheldon Wolff, Claire M. Berg, Roy Curtiss III (coauthor, Janet Renshaw), and Gustavo Cudkowicz

150th National Meeting of the American Chemical Society, Atlantic City, N.J. – W. E. Cohn (coauthor, Mayo Uziel)

Eleventh International Congress of Radiobiology, Rome, Italy – M. A Bender (coauthor, P. Carolyn Gooch)

Ninth Conference on Analytical Chemistry in Nuclear Technology, Gatlinburg, Tenn. – N. G. Anderson

International Conference on Murine Leukemia, Philadelphia – A. C. Upton (coauthors, V. K. Jenkins, H. E. Walburg, Jr., R. L. Tyndall, J. W. Conklin, and Niel Wald) and R. F. Zeigel (coauthors, R. L. Tyndall and Ernestine Teeter)

World Health Organization Conference on Chemistry and Physiology of the Gametes, Geneva, Switzerland – E. F. Oakberg

American Society for Cell Biology, Philadelphia – O. L. Miller, Jr., Tuneo Yamada (coauthor, Chinami Takata), and S. K. Brahma (coauthors, Piero Cammarano and Marialuisa Melli)

Sixteenth Annual Meeting of the Animal Care Panel, Philadelphia – J. T. Huffstetler (coauthor, M. L. Simmons), R. C. Allen (coauthors, R. T. Jones and L. J. Serrano), L. J. Serrano (coauthors, T. T. Odell, Jr., R. C. Allen, H. E. Walburg, Jr., and R. L. Tyndall), and A. C. Upton (coauthors, V. K. Jenkins, H. E. Walburg, Jr., R. L. Tyndall, J. W. Conklin, and Niel Wald)

Symposium on Transmission of Viruses by the Water Route, Cincinnati, Ohio – N. G. Anderson (coauthors, G. B. Cline and W. W. Harris)

Symposium on the Nucleolus, Its Structure and Function, Montevideo, Uruguay – Alexander Hollaender, O. L. Miller, Jr., and R. C. von Borstel

Foreign Travel

During this report period, 12 members of the Biology Division traveled to a total of 12 foreign countries, where they attended and participated in meetings and conferences, presented lectures and seminars, and consulted with other scientists. Countries visited were: Canada, Denmark, England, France, Germany, Hungary, Italy, Scotland, Sweden, Switzerland, The Netherlands, and Yugoslavia.

R. B. Setlow made two trips to Canada. On March 2, he presented a seminar at the University of British Columbia, Vancouver, entitled "A molecular error-correcting mechanism in DNA." In the second visit, July 20–23, he attended a conference on Effects of UV on Polynucleotides at the Department of Medical Biophysics, University of Toronto, Toronto, Ontario.

Liane B. Russell attended the CIBA Foundation Symposium on Preimplantation Stages of Pregnancy, April 13–15, in London, England, and presented a paper entitled "Death and chromosome damage from irradiation of preimplantation stages."

A. C. Upton attended the meeting of the International Commission on Radiological Protection and its committees on April 20–26 in Stockholm, Sweden, and presented a report on the radiobiological problems associated with the supersonic transport.

Dr. Upton served as chairman of a Task Group, appointed in April 1963, to study the biological effects of high-energy radiations, with special reference to cosmic rays and high-altitude flight.

While in Sweden, Dr. Upton also visited with Dr. Arne Nelson of the Research Institute of National Defence, Sundbyberg.

M. G. Hanna, Jr., and Takashi Makinodan traveled in Europe in April and May. Both attended the International Conference of Radiation and the Immune Response at the Chester Beatty Research Institute, London, and presented an invited paper. Dr. Hanna's paper was entitled "The role of lymphatic germinal centers in the immune response"; Dr. Makinodan presented "The effect of radiation on the immune condition."

After the London conference, Dr. Hanna conferred with Dr. R. G. White of the Bernhard Baron Institute and Dr. J. F. A. P. Miller of the Chester Beatty Research Institute. Then, on May 3, he visited with Dr. H. Cottier of the Institute of Pathology, University of Bern, Switzerland, and presented a seminar entitled "Lymphatic tissue changes during the primary immune response"; and on May 5–10, he conferred with Dr. D. W. van Bekkum of the Radiobiological Institute, National Research Council, Rijswijk (Z.H.), The Netherlands.

After presenting his paper in London (where he also participated in a conference discussion on immunological problems raised and solved by radiation chimeras), Dr. Makinodan visited Switzerland and Yugoslavia. On May 1–3, he attended the Symposium on Immunological Tolerance held in Lake Locarno, Switzerland, and presented an invited paper entitled "Cellular dynamics of immune response." Then on May 4–8, he accepted an invitation to visit with Dr. Zdenko Dizdar, Miroslav Simic, and colleagues in Belgrade and Zagreb, Yugoslavia, as a guest of the Institute of Nuclear Sciences "Boris Kidrich" in Belgrade and the Institute of Nuclear Sciences "Ruder Bošković" in Zagreb. He lectured on "Growth and senescence of the immune mechanism" while in Belgrade and Zagreb.

M. I. Dolin visited The Netherlands and France in June. On June 9, he attended the International Symposium on Flavins and Flavoproteins, and presented an invited paper, "NADH peroxidase," in Amsterdam, The Netherlands.

After the conference, he consulted with Drs. H. D. Peck and Jacquez Senez in Marseilles, France. Dr. Peck, a former member of the Biology Division Enzymology section, is now associated with the Department of Biochemistry, University of Georgia, Athens; Dr. Senez is with the Laboratoire de Biochemie Bactérienne, Marseilles.

Tuneo Yamada traveled to Sweden, England, Scotland, and Switzerland in May, June, and July. He was in Gothenburg, Sweden, May 17–June 16, to visit the Histological Institute at the University of Gothenburg, conduct a cooperative research program with Dr. Jan-Erik Edström on RNA of the lens regenerating system, and present an invited lecture "Behavior of lens specific antigens during lens regeneration." On June 18–19, he visited with Dr. H. B. Fell, Strangeways Research Laboratory, Cambridge University, Cambridge, England, and on June 20–27 was at the Institute of Animal Genetics, University of Edinburgh, Scotland, as a Macaulay Nonresident Fellow of the Institute. While at the Edinburgh Institute, he conferred with Professor C. H. Waddington, Drs. Ruth M. Clayton, G. G. Selman, Albert Jurand, and T. R. Elsdale.

Later, on July 8, after a brief vacation, he presented an invited lecture at the University of Zürich, Zürich, Switzerland. The title was "Cellular and subcellular events occurring during lens regeneration."

Alexander Hollaender traveled to The Netherlands, Hungary, Yugoslavia, Italy, and France during the month of June. In Amsterdam, he met with F. H. Sobels and discussed plans for the 1970 International Congress of Radiation Research meeting. In Budapest, Hungary, he visited four major installations. At the Institute of Radiation Biology and Radiation Hygiene he met with officials, participated in informal seminars, and visited the Departments of Pathophysiology, Microbiology and Immunology, Biochemistry, Morphology, Physics, and Radiation Hygiene. At the Institute of Genetics, he discussed the organization of Oak Ridge National Laboratory, met with officials, and again took part in informal seminars. At the Scientists Club of the Hungarian Academy of Science, he lectured on some of the research problems

being worked on in Oak Ridge. At the Institute of Medical Chemistry, he discussed problems of mutual interest and toured the facilities. He also met with officials of the Hungarian Atomic Energy Commission and the Ministry of Health.

At Pécs, Hungary, he met with J. Ernst, the head of the Institute of Biophysics of the University at Pécs, took part in discussions centered around international relations in biophysics, and gave a seminar. At Tihany, Hungary, he met with officials of the Biological Research Institute of the Hungarian Academy of Science, and toured the Departments of Experimental Zoology, Hydrobiology, and Experimental Botany.

In Belgrade, Yugoslavia, he met with Srdjan Hajdukovíc, the head of the Radiobiological Laboratory of the Institute "Boris Kidrich," took part in discussions, and gave seminars. He visited the Vinča Institute, and met with officials of the Yugoslav Nuclear Energy Commission. He also met with Salom Suica, the Secretary General of the Federal Nuclear Energy Commission.

At Zagreb, he visited the "Ruder Boškovíc" Nuclear Institute, participated in discussions, toured the facilities, gave a seminar, and toured the Cellular Radiobiology, Experimental Pathology, Bone Marrow Transplantation, and Health Physics Laboratories. At the "Jožef Stefan" Nuclear Institute in Ljubljana, he gave a seminar and toured the facilities.

In Rome, Italy, he met with G. E. Magni to discuss the meeting of the Third International Congress of Radiation Research to be held in Cortina d'Ampezzo on June 26–July 2, 1966. Dr. Hollaender is president of the Council of the International Association for Radiation Research. Dr. Hollaender met with Piero Cammarano, who was formerly associated with the Biology Division as a visiting investigator and is now with the Radiobiology Division, Atomic Energy Commission, at Casaccia. He also toured the facilities and held discussions with the director at Casaccia.

In Naples, Dr. Hollaender attended the Information Conference of the International Laboratory of Genetics and Biophysics at Naples, and met with G. Montalenti, Head of the Genetics Department of the University of Rome, and P. Pasquini, the official who represented the National Research Council. In Paris, Dr. Hollaender met with Martin Kaplan of the World Health Organization.

H. V. Malling made official visits to England, The Netherlands, Germany, and Denmark during June. In England, he visited Dr. Peter Brookes at the Chester Beatty Research Institute, London, to discuss the action of bifunctional compounds on DNA, and visited Dr. H. J. Evans at the Radiobiological Research Unit, Harwell, to discuss chromosome-breaking agents.

From England he traveled to Amsterdam to visit Drs. E. Kriek, N. S. Bond, I. J. Mizrahi, and P. Emmelot at the Netherland Cancer Institute. The subject of the discussion was the action of nitroso compounds on DNA and protein.

In Germany, Dr. Malling conferred with Drs. H. Druckery, R. Preussman, H. Marguarat, R. Schwaier, and F. K. Zimmerman at the University of Freiburg on (1) the mutagenic effect of nitroso compounds, and (2) antineoplastic compounds.

Dr. Malling then visited Copenhagen to confer with Dr. P. Feit at the Leo Pharmaceutical Factory, and to lecture to the Danish Microbial Society on June 22. The title of the lecture presented is "Mutagenic action of nitrous acid in *Neurospora* and the mutagenic action of antineoplastic compounds."

A. H. Warner visited the University of Windsor, Windsor, Ontario, Canada, June 17–18, and presented an invited seminar entitled "Metabolism of diguanosine nucleotides during brine shrimp embryogenesis."

Jane K. Setlow was in Toronto, Ontario, Canada, on July 21–23 to participate in discussions and attend a conference on the Molecular Basis of Ultraviolet Radiation at the University of Toronto.

I. L. Cameron left the Division on July 29 to attend the Second International Conference of Protozoology in London, England. While there, he presented a paper entitled "Regulation of the DNA-synthetic period within the cell cycle of *Tetrahymena pyriformis*" (coauthored by G. M. Padilla and Betty M. Wysinger), and conferred with Dr. E. C. Amoroso of the Royal Veterinary College.

Meetings and Conferences

TENTATIVE PROGRAM: NINETEENTH ANNUAL BIOLOGY RESEARCH CONFERENCE (1966)

The 1966 Research Conference, sponsored by the Biology Division of the Oak Ridge National Laboratory, will be held April 4–7 at the Riverside Motor Lodge in Gatlinburg, Tennessee. The organizing committee, Takashi Makinodan (chairman), J. F. Albright, F. T. Kenney, R. A. Popp, and Tuneo Yamada, have announced the following tentative program:

DIFFERENTIATION AND GROWTH OF HEMOGLOBIN AND IMMUNOGLOBULIN SYNTHESIZING CELLS

Monday, April 4

Morning

Chairman – V. M. Ingram, Department of Biology, Massachusetts Institute of Technology, Cambridge

Phylogenetic variation in the primary structure of hemoglobins – Gerhard Braunitzer, Max-Planck-Institut für Biochemie, München, Germany

Tertiary structure of hemoglobins and its functional significance – Makio Murayama, Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

Afternoon

Chairman – Felix Haurowitz, Department of Chemistry, Indiana University, Bloomington

Primary structure of immunoglobulins and its relationship to antibody specificity – Marian E. Koshland, Department of Molecular Biology, University of California, Berkeley

Subunits of immunoglobulins and their relationship to antibody specificity – R. R. Porter, Department of Immunology, Wright-Fleming Institute, St. Mary's Hospital Medical School, London, England

Tuesday, April 5

Morning

Chairman – George Klein, Institute for Tumor Biology, Karolinska Institutet, Stockholm, Sweden

Genetic regulation of peptide synthesis in hemoglobins – Harvey A. Itano, Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

Genetic regulation of immunoglobulin synthesis – Jacques Oudin, Service d'Immunochimie Analytique, Institut Pasteur, Paris, France

Afternoon

(Free)

Wednesday, April 6

Morning

Chairman – J. E. Till, Department of Medical Biophysics, University of Toronto, Toronto, Canada

Cytokinetics of antibody formation – Guy Sainte-Marie, Cancer Research Laboratory, The University of Western Ontario, London, Canada

Discussant – Miroslav Simic, Institute "Boris Kidrich," Belgrade, Yugoslavia

Cytokinetics and regulation of progenitor cells – L. G. Lajtha, Paterson Research Laboratories, Christie Hospital and Holt Radium Institute, Withington, Manchester, England

Afternoon

Chairman – Edward C. Horn, Department of Zoology, Duke University, Durham, North Carolina

Ontogenesis of the immune system – Donald Metcalf, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia

Ontogenesis of erythrocytes and hemoglobin formation – Corrado Baglioni, International Laboratory of Genetics and Biophysics, Naples, Italy

Thursday, April 7

Morning

Chairman – Edwin S. Lennox, The Salk Institute for Biological Studies, San Diego, California

Growth and senescence of antibody-forming cells – J. F. Albright, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Concluding remarks: the regulation of hemoglobin and immunoglobulin synthesis – Ray D. Owen, Biology Division, California Institute of Technology, Pasadena, and Claude Bennett, University of Alabama Medical School, University

PROCEEDINGS OF EIGHTEENTH ANNUAL BIOLOGY RESEARCH CONFERENCE (1965)

The Proceedings of the Eighteenth Annual Biology Research Conference, **Hormonal Control of Protein Biosynthesis**, held April 5–8, 1965, in Gatlinburg, Tennessee, are being published as an October supplement to the *Journal of Cellular and Comparative Physiology*.

TENTATIVE PROGRAM: SYMPOSIUM ON THE NUCLEOLUS, ITS STRUCTURE AND FUNCTION

The **Symposium on the Nucleolus, Its Structure and Function** will be held December 5–10, 1965, at the Facultad de Medicina, Universidad de Montevideo, at Montevideo, Uruguay.

Proposed sponsors include Biology Division, Oak Ridge National Laboratory; city of Montevideo; Institute for Cancer Research, Philadelphia; National Council of Scientific and Technical Research of Uruguay; Organization of American States; United States Atomic Energy Commission; United States National Science Foundation; University of Montevideo; and University of Pittsburgh, Pittsburgh, Pa.

Symposium Committee officers are Honorary President Clemente Estable, Instituto de Investigacion de Ciencias Biologicas, Montevideo; President Alexander Hollaender, Biology Division, Oak Ridge National Laboratory; and Vice-President Francisco A. Saez, Instituto de Investigacion de Ciencias Biologicas, Montevideo.

Serving as committee members are M. E. Drets, Instituto de Investigacion de Cienicas Biologicas, Montevideo; Mary Esther Gaulden, Southwestern Medical School, University of Texas, Dallas; G. Gerard, Facultad de Humanidades y Ciencias, University of Montevideo; O. L. Miller, Jr., Oak Ridge National Laboratory; Crodowaldo Pavan, University of São Paulo; R. P. Perry, Institute for Cancer Research, Philadelphia; H. Poletti, Facultad de Medicina, University of Montevideo; J. R. Sotelo, Instituto de Investigacion de Ciencias Biologicas, Montevideo; J. I. Valencia, University of Buenos Aires; W. S. Vincent, University of Pittsburgh; and R. C. von Borstel, Oak Ridge National Laboratory.

The following is the tentative program:

SYMPOSIUM ON THE NUCLEOLUS, ITS STRUCTURE AND FUNCTION

Sunday, December 5

Evening

Session I – Introduction

Chairmen – Clemente Estable, Instituto de Investigacion de Ciencias Biologicas, Montevideo

– J. R. Totter, Division of Biology and Medicine, USAEC, Washington, D.C.

Speaker – Jean Brachet, Université Libre, Bruxelles, Belgium

Monday, December 6

Morning

Session II – Nucleolar Structure I

Chairmen – Lucien Lison, University of São Paulo, Ribeirao Preto, Brazil

– J. H. Taylor, Florida State University, Tallahassee

Paper – Hewson Swift, University of Chicago

Discussants – Max Alfert and N. K. Das, University of California, Berkeley

– Ruth G. Kleinfeld, Syracuse University, Syracuse, N.Y.

– Robert Love, Jefferson Medical College, Philadelphia

– C. J. Tandler, Instituto Fitotechnico de Santa Catalina, Llavallol, Argentina

Afternoon

Session III – Nucleolar Structure II

Chairmen – E. D. P. De Robertis, University of Buenos Aires, Argentina

– Jack Schultz, Institute for Cancer Research, Philadelphia

Paper – William Bernhard, Institute for Cancer Research, Villejuif, France

Discussants – L. A. Chouinard, Laval University, Quebec, Canada
– Nicole Granboulan, Institute for Cancer Research, Villejuif, France
– O. L. Miller, Jr., Oak Ridge National Laboratory
– Unne Stenram, University of Uppsala, Uppsala, Sweden

Evening

9:00 PM – Reception

Tuesday, December 7

Morning

Session IV – Nucleolar Composition
Chairmen – Carlos Basilio, University of Chile, Santiago
– P. P. Cohen, University of Wisconsin, Madison
Papers – J.-E. Edström, University of Göteborg, Göteborg, Sweden
– Harris Busch, Baylor University, Houston
Discussants – D. G. Comb, Harvard University, Cambridge, Mass.
– J. G. Gall, Yale University, New Haven, Conn.
– Rachele Maggio, University of Palermo, Italy
– Günther Siebert, Johannes Gutenberg University, Mainz, Germany

Afternoon

Session V – Nucleolar Genes I
Chairmen – Giorgio Schreiber, University of Minas Gerais, Belo Horizonte, Brazil
– Barbara McClintock, Carnegie Institution of Washington, N.Y.
Papers – Clemente Estable, Instituto de Investigacion de Ciencias Biologicas, Montevideo
– Claus Pelling, Max-Planck-Institut, Tübingen, Germany
– F. M. Ritossa, University of Illinois, Urbana, and LIGB, Naples
Discussants – H. J. Barr, University of Wisconsin, Madison
– J. G. Lafontaine, Laval University, Quebec, Canada
– Arlene C. Longwell, Children's Cancer Research Hospital, Boston
– J. R. Sotelo, Instituto de Investigacion de Ciencias Biologicas, Montevideo

Evening

Session VI – Nucleolar Genes II
Chairmen – Crodowaldo Pavan, University of São Paulo, Brazil
– A. A. Buzzati-Traverso, International Laboratory of Genetics and Biophysics, Naples

Papers – E. H. McConkey, Harvard University, Cambridge, Mass.
– M. L. Birnstiel, University of Edinburgh, Edinburgh, Scotland
Discussant – Hugh Wallace, Amherst College, Amherst, Mass.

Wednesday, December 8

(Free)

Thursday, December 9

Morning

Session VII – The Nucleolus and Ribosome Synthesis
Chairmen – Henrique Tono, Universidad del Valle, Cali, Colombia
– C. P. Swanson, Johns Hopkins University, Baltimore
Papers – G. P. Georgiev, Academy of Sciences, USSR
– R. P. Perry, Institute for Cancer Research, Philadelphia
– J. E. Darnell, Yeshiva University, New York

Afternoon

Session VIII – Nucleolar Activities
Chairmen – M. E. Drets, Instituto de Investigacion de Ciencias Biologicas, Montevideo
– I. B. Zbarsky, Academy of Sciences, USSR
Papers – W. S. Vincent, University of Pittsburgh, Pittsburgh, Pa.
– J. L. Sirlin, University of Edinburgh, Edinburgh, Scotland
Discussants – Adrienne Ficq, Université Libre, Bruxelles, Belgium
– C. P. Leblond, McGill University, Montreal, Canada
– P. S. Woods, University of Delaware, Newark

Friday, December 10

Morning

Session IX – The Nucleolus in the Cell Cycle and During Development
Chairmen – J. I. Valencia, University of Buenos Aires, Buenos Aires, Argentina
– R. C. von Borstel, Oak Ridge National Laboratory
Papers (*a*) – Mary Esther Gaulden, Southwestern Medical School, Dallas, Tex.
– L. E. Feinendegen, Laboratoire Pasteur, Paris
– Renate Lettré, University of Heidelberg, Heidelberg, Germany

(*b*) – D. D. Brown, Carnegie Institution of Washington, Baltimore
– Beatrice Mintz, Institute for Cancer Research, Philadelphia
– Marko Zalokar, University of California, San Diego

Afternoon

Session X – Closing Session
Chairman – F. A. Saez, Instituto de Investigacion de Ciencias Biologicas, Montevideo
Speaker – C. H. Waddington, University of Edinburgh, Edinburgh, Scotland

ANNUAL INFORMATION MEETING SCHEDULED

The Oak Ridge National Laboratory Advisory Committee for Biology will make its annual visit to the Division November 17, 18, and 19. The committee is composed of C. E. Carter, Western Reserve University Medical School; Erwin Chargaff, Columbia University; Rollin D. Hotchkiss, The Rockefeller Institute; Henry S. Kaplan, Stanford University Medical School; Earl R. Stadtman, National Heart Institute; and Curt Stern, University of California.

During this visit, Division investigators will summarize their work for the visitors at a conference in the Division, and the committee members will visit individual laboratories and participate in informal discussions.

BIOMEDICAL DIRECTORS MEETING

Alexander Hollaender attended the spring meeting of the Biomedical Program Directors of the United States Atomic Energy Commission at Argonne National Laboratory, Chicago, on May 10–11. Meetings of this type are held three times each year to review research in the various biomedical programs at Commission laboratories.

BONE MARROW CONFERENCES

December Conference Planned – The latest in the series of bone marrow and chemical protection conferences, the first of which was held in 1957 in Oak Ridge, is scheduled for December 2–3 at the United States Naval Radiological Defense Laboratory, Hunter Point, San Francisco.

Session topics tentatively include the following: (1) chemical protection against radiation injury and injury from toxic chemicals, (2) bone marrow transplantation, (3) immunotherapy of cancer, (4) cell separation (hemopoietic and lymphatic tissues), (5) stem cells (hemopoietic and lymphatic tissues), and (6) experimental hematology.

April Conference Held – Two papers were presented by Division investigators at the Discussion Meeting on Bone Marrow Transplantation and Chemical Radiation Protection, which was held April 10 at Atlantic City, New Jersey. These were: "Reduced mortality from secondary disease in radiation chimeras," by C. C Congdon, M. A. Kastenbaum, and D. A. Gardiner, and "Spleen weight as a measure of bone marrow cell growth: comparisons with spleen colony formation and ^{59}Fe uptake," by R. A. Popp, C. C Congdon, and Joan Wright Goodman.

The conference was organized by L. J. Cole of the United States Naval Radiological Defense Laboratory and by C. C Congdon.

Other Future Meetings – Plans for future meetings include an evening session at the time of the Federation meetings in Atlantic City, New Jersey, April 12, 1966, and a special conference on Germinal Centers of Lymphatic Tissue to take place in Bern, Switzerland, June 22–24, 1966, just prior to the Third International Congress of Radiation Research in Cortina d'Ampezzo, Italy. H. Cottier, Universität Bern, is arranging the germinal center meeting.

Issues of Experimental Hematology – Two issues (Nos. 7 and 8) of *Experimental Hematology* were published during this report period. The first contains abstracts of material presented at the three 1964 conferences; the second includes abstracts from the April 1965 conference held in Atlantic City.

Future issues will be published within three months of each meeting held. *Experimental Hematology* is edited and organized in the Division, with the assistance of the Technical Publications Department of the ORNL Technical Information Division.

Educational Activities

STUDENT TRAINEE PROGRAM

During the summer months 18 students between their junior and senior years at undergraduate colleges, principally in the Southeast, worked under the direct supervision of a Biology Division senior investigator. Each student was required to present a final oral and written report of his summer's work.

The 1965 student trainees, their affiliations, and supervisors and groups to which they were assigned were:

STUDENT TRAINEE	INSTITUTION	ASSIGNED TO
Bruce Stewart Baker	Reed College, Portland, Ore.	R. B. Setlow, Biophysics
Myra Berman	Goucher College, Towson, Baltimore	T. R. Manney, Cytochemistry and Cell Reproduction
Martha Copley	Hood College, Frederick, Md.	A. P. Harrison, Jr., Chemical Carcinogenesis
Diane Marie Cottingham	Our Lady of the Lake College, San Antonio, Tex.	R. A. McGrath, Biophysics
Joan Denise Edwards	Barber-Scotia College, Concord, N.C.	A. H. Haber, Plant Physiology
Anne Steele Faust	Wilson College, Chambersburg, Pa.	F. T. Kenney, Enzymology
Sue Ellen Frederick	Glenville State College, Glenville, W.Va.	R. C. von Borstel, Cytochemistry and Cell Reproduction
William Pressly Hunt	Erskine College, Due West, S.C.	Peter Pfuderer, Enzymology
Marianne Louise Jefferson	Maryville College, Maryville, Tenn.	T. T. Odell, Jr., Pathology and Physiology
Louis Victor Kaufman	Wheeling College, Wheeling, W.Va.	L. H. Smith, Mammalian Recovery

STUDENT TRAINEE	INSTITUTION	ASSIGNED TO
David Charles Levin	Reed College, Portland, Ore.	Lawrence Rosen, Enzymology
Charlotte Mae Mistretta	Trinity College, Washington, D.C.	W. D. Gude, Pathology and Physiology
Carolyn Gene Mohler	Longwood College, Farmville, Va.	F. J. Finamore, Nucleic Acid Enzymology
Sarah Dianne Phillips	Winthrop College, Rock Hill, S.C.	C. C Congdon, Mammalian Recovery
Thomas Pitner	College of Charleston, Charleston, S.C.	M. L. Randolph, Biophysics
Margaret Langhorne Rachal	Randolph-Macon Woman's College, Lynchburg, Va.	Waldo E. Cohn, Nucleic Acid Chemistry
Ruth Elsovene Thomas	Pfeiffer College, Misenheimer, N.C.	P. A. Roberts, Drosophila Cytology and Genetics
Ina Kaye Wachsmuth	Stetson University, DeLand, Fla.	C. J. Wust, Enzymology

Fourteen other students who were not under the Student Training Program were selected to train in the same manner. These students have completed work for their B.S. degrees, and will be in graduate school in September 1965. They were:

NAME	INSTITUTION	ASSIGNED TO
William Leonard Albritton	Medical College of Alabama, Birmingham	F. T. Kenney, Enzymology
David Eugene Axelrod	University of Tennessee, Knoxville	H. I. Adler, Radiation Microbiology – Bacterial Genetics
Carlos Clinton Campbell III	Haverford College, Haverford, Pa.	Lawrence Rosen, Enzymology
Patricia Gail Carver	University of Tennessee, Knoxville	Virginia P. White, Administrative Office
James Lon Everett	Auburn University, Auburn, Ala.	M. L. Simmons, Experimental Animal Facility
William Cullen Grant	Livingston College, Salisbury, N.C.	C. C Congdon, Mammalian Recovery
Barbara Hamkalo	University of Massachusetts, Amherst	R. B. Setlow, Biophysics
Suzanne Holt	Agnes Scott College, Decatur, Ga.	Virginia P. White, Administrative Office
Jonathan Kaplan	University of Pennsylvania Medical School, Philadelphia	C. C Congdon, Mammalian Recovery

NAME	INSTITUTION	ASSIGNED TO
Fred Albert Mettler	Columbia University, New York	W. D. Fisher, Subcellular and Viral Physiology
Jane Brodie Mills	Columbia University, New York	M. P. Stulberg, Enzymology
Janet Renshaw	Vanderbilt University, Nashville, Tenn.	Roy Curtiss III, Radiation Microbiology
William Robert Somers	Duke University, Durham, N.C.	R. A. Brown, Radiation Immunology
Billy Joe Williams	University of Tennessee, Knoxville	D. L. Lindsley, Drosophila Cytology and Genetics

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, five Division investigators presented lectures on this program. They include: D. E. Foard on March 13–14 at Spring Hill College, Mobile, Alabama, "The cell and the organism"; T. T. Odell, Jr., on March 22 at Western Kentucky State College, Bowling Green, "Regulation of blood platelets"; O. L. Miller, Jr., on April 19 at Winthrop College, Rock Hill, South Carolina, "Electron microscopy of chromosomes"; R. L. Tyndall on April 20–22 at Western Maryland College, Westminister, "Viruses, a continuing challenge" (this lecture was presented three times); and L. J. Serrano on April 25–26 at Georgia Women's College, Milledgeville, "Animals in research."

UNIVERSITY COOPERATIVE PROGRAM

Two Division members presented lectures at the University of Georgia, Athens, during this report period. G. D. Novelli presented a lecture on "Protein synthesis by regenerating liver" to graduate students in biochemistry on February 26. Later, S. F. Carson was in Athens on May 16–17 to give two lectures to the Bacteriology Department. The lecture titles were: "Biotin and B_{12} coenzyme catalyzed reactions in the interconversion of propionate and succinate: (1) Propionyl carboxylase and the transcarboxylases, (2) Methylamalonyl isomerase and racemase."

H. I. Adler and A. H. Haber are continuing their association with the University of Tennessee in part-time teaching and advisory capacities under the joint UT–Oak Ridge National Laboratory Cooperative Research Program.

During the academic year, Dr. Adler has been lecturing on bacterial genetics for the University's Department of Bacteriology. Roy Curtiss III presented a lecture on "Bacterial lysogeny" at two sessions during this course.

SCIENCE FAIR

S. F. Carson was a member of the panel of 12 judges named by United States Atomic Energy Commission Chairman Glenn T. Seaborg to select winners of AEC special awards at the 16th National Science Fair-International May 5 in St. Louis.

Earlier, Dr. Carson was guest speaker at the Awards Dinner of the 13th Annual Southern Appalachian Science Fair. The event was held at the University Center, University of Tennessee, Knoxville, on

March 26. Dr. Carson spoke on "Modern experimental biology: the synthesis of mathematics, physical science and biology."

OAK RIDGE NATIONAL LABORATORY UNDERGRADUATE LECTURE PROGRAM

E. H. Y. Chu presented two lectures under the Undergraduate Lecture Program during this report period. On March 14–15 at Converse College, Spartanburg, South Carolina, he presented "Cytogenetic effects of radiation in mammalian cells grown in culture" and "Human cytogenetics."

TRAINING SESSION HELD AT VIRUS SEPARATION FACILITY

A training session and symposium on new separation systems and experimental studies were held May 20–22 in the recently completed virus isolation facility at ORNL's Biophysical Separations Laboratory. N. G. Anderson is coordinator of the facility.

A total of 65 scientists from this country and abroad participated in these meetings.

The new facility – adjacent to the Oak Ridge Gaseous Diffusion Plant – has three primary aims: to develop new centrifuge systems for cell fractionation and for human vaccine production; to make purified viruses available for medical research; and to train scientists in new separation methods.

The three-day meeting, sponsored by ORNL's Office of Industrial Cooperation, was particularly aimed at biological scientists and interested technical representatives from universities, research laboratories, and pharmaceutical and manufacturing firms.

ORNL's Biophysical Separations Laboratory is jointly sponsored by the National Cancer Institute, the National Institute of Allergy and Infectious Diseases, and the AEC.

Biology Division members who presented lectures include N. G. Anderson ("Introduction to zonal centrifugation," "S-ρ techniques," "Continuous flow rotors," and "New experimental rotor systems"); G. B. Cline ("Continuous flow centrifugation with banding" and "A review of experiences in large scale virus isolation"); W. D. Fisher ("B-X rotor system" and "Isolation of macroglobulins and ribosomes"); and A. A. Barber ("Isolation of native glycogen by combined rate and isopycnic centrifugation").

INTRODUCTORY COURSE IN MODERN ZOOLOGY

The "Introductory Course in Modern Zoology," organized by the Division's G. L. Whitson and N. G. Anderson, has completed its first year. The course was taught at the Oak Ridge Institute of Nuclear Studies in cooperation with the Biology Division and the University of Tennessee in order to train Oak Ridge nonbiological personnel in basic biology.

Division members who presented lectures in the spring quarter include: W. D. Fisher, "Research in biophysics, isolation of subcellular particles"; Peter Mazur, "The role of water, permeability, and temperature in living cells"; T. R. Manney, "Gene control of enzyme formation"; A. C. Upton, "Radiation and cancer"; Roy Curtiss III, "Mechanism of genetic recombination in bacteria"; F. J. Finamore, "Recent advances in the biochemistry of development"; W. A. Arnold, "Problems concerning the mechanism of photosynthesis"; G. L. Whitson, "Problems in cell division synchrony" and "Morphogenesis in ciliated protozoa"; and R. C. von Borstel, "Developmental genetics, gene control during growth, and differentiation."

The course will be offered again in 1965–66 for students who have either an A.B. or B.S. degree. University credit of four hours per quarter may be earned on the undergraduate level (301, 302, and 303 zoology), and will allow students who complete the course to take other University of Tennessee 300-level courses in biology and biochemistry (assuming chemistry requirements are met). The course may be taken for graduate credit by nonbiology majors only. Topics in each of the courses include: 301 – levels of biological complexity, ecology; 302 – reproduction, growth, cell differentiation, and genetics; and 303 – subcellular organization.

PARTICIPATION IN OTHER COURSES

Lectures – W. D. Gude presented two lectures as part of the Visiting Scientists Program of the Tennessee Academy of Science at Lanier High School, Maryville, Tennessee, on March 2. Titles were: "Biological effects of radiation in mammals" and "Autoradiography as a tool in research."

W. L. Russell lectured on "The scientific method" on April 30 on Tennessee Junior Science Day, an event sponsored by the Tennessee Academy of Science, Union Carbide Corporation, and the Oak Ridge Institute of Nuclear Studies.

Liane B. Russell and G. E. Cosgrove gave lectures at the Oak Ridge School of Reactor Technology on June 10 and June 11 respectively. Dr. Cosgrove's subject was "Delayed effects of radiation in mammals," and Dr. Russell's title was "Genetic effects of radiation."

G. E. Cosgrove spoke on "Biological effects of radiation" at the Oak Ridge National Laboratory Radiation Safety Training Program on June 25.

Three Division members lectured in the Marine Biological Laboratory's embryology training program at Woods Hole, Massachusetts, during June and July. R. A. Wallace lectured on "Biomedical aspects of vertebrate yolk formation and structure" on July 19; Alexander Hollaender presented "Studies in radiation biology at Oak Ridge National Laboratory" on July 16; and J. F. Albright lectured on "Competence of cells for antibody formation" on July 18.

Four Division members lectured in a University of Tennessee summer course, "Survey of General Pathology," offered at the Special Training Division of the Oak Ridge Institute of Nuclear Studies during June and July. On June 17, W. D. Gude lectured on "Pathology defined – nature of disease" and "Degeneration." On June 21, N. K. Clapp gave two lectures on "Circulatory disturbances" and on June 28 two lectures on "Growth and development." On July 1, A. C. Upton gave two lectures on the "Pathology of aging," and on July 19 he lectured twice on the "Pathology of radiation." G. E. Cosgrove gave two lectures on "Infection: bacterial, viral, parasitic" on July 8.

C. M. Steinberg taught a section of the Bacterial Viruses course offered at Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, July 3–August 6.

Courses Taken – Amir Muhammed attended the course on Bacterial Viruses at Cold Spring Harbor from July 7 to August 5.

Division Members on Leave of Absence

Ernest Hsiao-Ying Chu has been awarded a National Science Foundation Senior Postdoctoral Fellowship to spend one year at the Karolinska Institute, Stockholm, Sweden, where he will be associated with Dr. George Klein. Dr. Chu will be in Sweden from September 1965 through September 1966.

D. L. Lindsley has been awarded a National Science Foundation Senior Postdoctoral Fellowship to spend one year at the Instituto di Genetica in Rome, Italy, where he will be in the laboratory of Professor G. Montalenti. Dr. Lindsley will be in Italy from September 1965 through August 1966.

Honors and Appointments

UPTON RECEIVES E. O. LAWRENCE AWARD

A. C. Upton was one of five persons named by United States Atomic Energy Commission Chairman Glenn T. Seaborg to receive the Ernest Orlando Lawrence Memorial Award for 1965. The award is made annually to honor scientists for recent meritorious contributions to the field of atomic energy. Dr. Upton was cited "for outstanding contributions to radiobiology and to the pathology of radiation injury."

Dr. Upton, a member of the Biology Division since 1951, is considered one of the world's leading radiation pathologists. His work deals with pathological processes induced by radiation exposure, both in man and in animals. He has demonstrated transmissible chromosome aberrations in leukemic mice and has correlated the finding with the occurrence of latent viruses made active by irradiation.

The award was established by the Commission in 1959 in honor of the late E. O. Lawrence, inventor of the cyclotron and director of the radiation laboratory at the University of California, Berkeley and Livermore, which bears his name.

COHN TO DIRECT NOMENCLATURE OFFICE

Waldo E. Cohn has been appointed director of the newly established Office of Biochemical Nomenclature for the National Academy of Sciences – National Research Council. He will be serving on a part-time basis.

The new office is designed to improve communication among individuals and groups concerned with the problem of assigning names to the large and rapidly growing list of biochemical compounds. It will seek to coordinate information on the activities of national and international organizations in biochemical nomenclature, to stimulate new activities as deemed desirable, and to encourage the dissemination of information to interested groups.

Dr. Cohn is currently the secretary of the Combined Commission on the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. In this capacity he will provide direct liaison between the new office and the nomenclature authorities of the national scientific bodies represented in the two Unions.

Dr. Cohn has been associated with the Division since 1947.

Cytology and Genetics

R. F. Kimball[a]

Effects of Radiation on Paramecium (1.1–1.4)

R. F. Kimball[a]
D. Q. Brown[b]
Satomi Tgarashi[c]
G. L. Whitson[d]
E. G. Bailiff[a]
Barbara R. Bussell[a]
Stella W. Perdue
Carrie L. Wells[a]

Phage Genetics (1.5–1.8)

Jane K. Setlow
C. M. Steinberg
Amir Muhammed[c]
M. E. Boling

Mammalian Genetic Cytology (1.9, 1.10)

E. H. Y. Chu[a]
J. D. Regan[d]
J. E. Trosko[a,e]
Lana T. Covington
Margaret R. Kasschau[a]
Nina S. Hammer[a]
Kiki Hellman[f]

Mammalian Biochemical Genetics (1.11–1.13)

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

***Rhynchosciara* Genetics (1.14–1.17)**

Crodowaldo Pavan[c]
Renato Basile[c]
Shirley A. Preston

Participation in Cooperative Projects:

NIH-AEC – Carcinogenesis Programs (24.9, 24.12)

[a]Dual assignments.
[b]Research participant.
[c]Visiting investigator from abroad.
[d]USPHS Fellow.
[e]American Cancer Society Fellow.
[f]Consultant.

1.1 STUDIES ON PERSISTENT INTRACLONAL VARIATIONS IN CELL MASS AND CELL GROWTH RATE IN *PARAMECIUM AURELIA*

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

In the previous semiannual report[1] we showed that different cell lineages within a clone of *Paramecium aurelia* may differ in total cell mass, that these differences may persist for a number of cell generations, and that there is a positive correlation between cell mass and macronuclear DNA content. These findings have been amply confirmed, and we have begun an analysis of the factors controlling these persistent intraclonal variations.

Methods. – The dry mass of individual cells was measured by the Caspersson scanning microinterferometer, and the macronuclear DNA content by Feulgen microspectrophotometry with the Zeiss scanning microspectrophotometer.

The general procedure, varying in details from experiment to experiment, was to establish between 20 and 60 cell lineages with single-cell isolates and to continue them by daily single-cell isolations. In some cases, part of the cells from

a lineage were allowed to exhaust the food or to undergo the self-fertilization process of autogamy and were then returned to normal growth conditions. Other cells of the lineage were maintained continuously in normal log-phase growth. On each of two or more different days, one or more cells from each lineage and its starved or exautogamous sublineages were dried on slides. These cells were always dried immediately after division to obtain a standard stage in the cell cycle for measurement. Maps were kept of the position of each cell on the slide so that its lineage and treatment could be identified. The slides were first measured, unfixed, by microinterferometry and were then fixed and stained by the Feulgen procedure and measured by microspectrophotometry.

Results. – The data show that cells within a lineage continue to be more alike in cell mass and DNA content than cells in different lineages for at least eight days and 34 cell generations. The evidence also shows that this similarity becomes less marked with time. In other words, cell mass and DNA content are inherited for many cell generations but not indefinitely. The mass differences persist, at least in part, through starvation by gradual exhaustion of the medium and through the self-fertilization process of autogamy. There is some evidence, though incomplete, that the mass differences do not persist through rapid starvation brought about by transfer to nonnutrient medium.

Not only has the correlation between mass and DNA content been amply confirmed, but also a positive correlation with division rate has been found. Cell lineages with a greater mass and DNA content at division also tend to have a greater division rate. When both the mass at division and the division rate are taken into account, it can be calculated that different cell lineages may differ by as much as a factor of 2, perhaps more, in the amount of protoplasm synthesized per unit time. Thus the differences in growth rate within a clone may be quite appreciable.

Discussion. – The data as yet are not adequate for developing a hypothesis about the physical basis for these persistent differences in cell mass and growth rate, but certain possibilities can be excluded. For example, random variations in macronuclear size could arise through unequal amitotic division[1] and lead to unequal growth rates. Such a phenomenon could explain much of the data but not the persistence through autogamy, when a new macronucleus is developed by endomitotic growth from the diploid synkaryon. Indeed, the persistence through autogamy suggests that macronuclear size itself may be determined by whatever is responsible for the growth rate determination, since the size of the newly formed macronucleus must be determined in part by the preexisting size. It is also clear that a regular cycle of growth and division is not essential for maintaining the size characteristics of the cell lineages, since these characteristics are maintained through gradual starvation and autogamy, both of which interrupt the normal growth cycle and cause major, though temporary, departures from normal cell size. On the other hand, somewhat incomplete evidence suggests that a period of rapid starvation prevents the maintenance of the preexisting size differences and allows a new set of differences, random with respect to the old ones, to be established. This leads to some hope that experimental intervention will allow an analysis of the mechanisms that control such important cell properties as the growth rate and the amount of major cellular constituents.

References

[1]R. F. Kimball, Stella W. Perdue, and E. G. Bailiff, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1965*, ORNL-3768, p. 36.

1.2 MODIFICATION OF *TS* MUTATIONS BY PRE- AND POSTIRRADIATION CONDITIONS IN *PARAMECIUM AURELIA*

Satomi Igarashi

Numerous studies on the mechanism of mutagenesis in *P. aurelia* have been carried out, using various modifying conditions.[1] Parallel experiments to the earlier ones with ordinary lethal mutations have now been carried out with a new class of mutations[2,3] – temperature-sensitive mutations (*ts*) that can grow normally at 27°C but are inviable at 36°C – with similar results.

Stage of Cell Division Cycle. – Paramecia in the preduplication interphase (G_1 period) are much more sensitive to x-ray-induced ordinary mutation than those in the postduplication interphase (G_2 period).[1] X irradiation (2.5 kr) of specimens in G_1 yields approximately 20%, and irradiation in G_2 approximately 2% *ts* mutant segregants. This

result is exactly the same as that for ordinary lethal mutagenesis.

Stage of Culture Growth Cycle. – At the first postautogamous division, 80 specimens were incubated in a dish containing 10 ml of culture medium. At 12-hr intervals thereafter, relative cell size and interdivision intervals were measured for each 10 sample specimens, and then 50 specimens at G_1 period were irradiated with 2.5 kr. Relative cell size decreased gradually, while the interdivision interval decreased from 12 hr in the first 12 hr of incubation to 6 hr during the next 24 hr and then increased again up to 12 hr for the last cell generation prior to stationary phase. The frequency of *ts* mutant segregants is directly related to the interdivision intervals but not to growth stage or to cell size. The shorter the interdivision intervals, the higher the mutation yield.

Postirradiation Treatment with Streptomycin. – Dividing specimens were collected and irradiated with 2.5 kr of x rays as usual. Irradiated specimens were divided into two groups, one for the irradiated control and the other for streptomycin treatment. The latter group was transferred into a streptomycin solution containing 3 mg of streptomycin per milliliter of culture medium and kept for 4 hr. Sets showing any mortality during treatment were discarded. Streptomycin treatment following irradiation decreased the yield of *ts* mutant segregants, as was found before with the ordinary lethal mutations.[1]

Effect of Postirradiation Starvation. – In the earlier work[1] it was reported that the longer the time between irradiation and refeeding of stationary-phase paramecia, the less the mutation yield. Tests were performed to determine whether the same thing is true for *ts* mutations. Paramecia were kept in small amounts of culture medium for four days after autogamy to obtain starved nonautogamous specimens. These starved specimens were irradiated with 5 kr of x rays. Immediately after irradiation, 50 samples were isolated into fresh culture medium and kept by daily isolation method. The other two sample groups were transferred into fresh medium 12 and 24 hr after irradiation. The shorter the time between irradiation and refeeding, the higher the *ts* mutation yield. This correlation confirms that found for ordinary mutations in the earlier work.[1]

Conclusions. – The effects of the stage in the cell division cycle, postirradiation treatment with streptomycin, and postirradiation starvation on the yield of *ts* mutants closely parallel the previous results with ordinary lethal mutations.[1] The same interpretation seems applicable. At least part of the mutations arise from reparable premutational damage that is converted to mutation at chromosome replication. The longer the time between irradiation and replication, the more repair. The studies on the effect of the culture growth cycle are new but can be interpreted in the same way. Thus, at least part of the *ts* and ordinary lethal mutations are produced in basically the same way.

References

[1]R. F. Kimball, *J. Cellular Comp. Physiol.* **58**, suppl. 1, 163 (1961).

[2]S. Igarashi and R. F. Kimball, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1964,* ORNL-3601, p. 30.

[3]S. Igarashi, *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1964,* ORNL-3700, p. 36.

1.3 TEMPERATURE-SENSITIVE CATALASE OF A *TS* MUTANT AND ITS INHERITANCE

Satomi Igarashi

Direct evidence of molecular changes by mutation has been sought after finding a new class of mutation,[1] a temperature-sensitive mutation (*ts*), in *Paramecium aurelia.* Temperature sensitivity of the catalase reaction of brei preparations from *ts* mutant clones was tested as one possible way of finding such changes.

Out of about a hundred *ts* mutants tested, stock 2149 was found to have a temperature-sensitive catalase reaction. This strain was crossed to wild type twice, and its derivatives were kept in stock, especially *ts* derivative 41. As a control, other *ts* mutants and wild-type stocks were used.

Cell-free brei was prepared by passing about 100,000 specimens through a syringe of 1-ml capacity five times. Then catalase activity was checked by the method given by Sumner and Dounce,[2] and permanganate consumption by 5-ml samples of reaction mixture, a sample being withdrawn every 5 min after incubation began, was plotted semilogarithmically.

Brei preparations of wild-type paramecia did not show first-order kinetics of the catalase reaction. At 35.5°C, the first 5 min of incubation showed

a very fast *reaction 1*; thereafter a relatively slow *reaction 2* appeared, which is the first-order reaction. At 50°C, *reaction 1* occurred, but no *reaction 2* was detected. In addition, the experiment in which the reaction mixture without peroxide was incubated at 50°C for 15 min and then peroxide was added showed that *reaction 1* remained active. Eventually it could be assumed that *reaction 1* is heat insensitive, perhaps a mixture of nonenzymatic and enzymatic decomposition of peroxide, while *reaction 2* is heat labile, presumably enzymatic. This kind of reaction pattern seems to be caused by the use of crude brei preparations.

In *ts* mutant stock 2149, derivative 41, *reaction 2* was prevented by incubation at 35.5°C, unlike the wild type. The other features at 27 and 50°C were the same as in wild-type paramecia. The other *ts* mutant stocks did not show this characteristic. Therefore, this reaction pattern at 35.5°C suggests a heat-labile catalase in this mutant.

The heat lability of brei preparation of the F1 and F2 generations was tested in crosses. In the cross between derivative 41 of stock 2149 and wild type the F1 generation showed the reaction pattern of wild type at 35.5°C. In the F2 generation, out of 76 segregants tested, the 30 *ts* segregants showed heat lability of *reaction 2* at 35.5°C but a normal reaction at 27°C. The 46 wild-type segregants showed the normal wild-type pattern. The *ts* segregants from crosses of other *ts* mutants showed the wild-type catalase reaction.

The crude method in the present experiment leaves some uncertainties in the detection of heat lability of the catalase reaction. However, qualitatively, it seems clear enough that the temperature sensitivity of the mutant stock 2149 is caused by the temperature sensitivity of its own catalase and that this heat lability of catalase is controlled by a mutated gene. Therefore, it may be concluded that the temperature-sensitive mutation could result from the production of an altered enzyme which is normal at lower temperatures but labile at higher temperatures. It seems possible that the other *ts* mutations are caused by the alterations of other enzymes than catalase.

References

[1]S. Igarashi and R. F. Kimball, *Genetics* **50**, 258 (1964).

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1.4 EFFECT OF ACTINOMYCIN D ON THE POLYSOMES OF SYNCHRONIZED *TETRAHYMENA*

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

Introduction. – This report concerns the formation of polyribosomes, subsequent inhibition by actinomycin D, and these relations to stomatogenesis and cell division in temperature-synchronized *Tetrahymena*. The B-IV zonal ultracentrifuge designed by Anderson *et al.*[2] was used for the isolation of ribosomes in this investigation.

Methods. – Cells were synchronized by a repetitive temperature cycle as previously described by Padilla and Cameron.[3] This cycle consists of 9.5 hr at 12°C and 2.5 hr at 27°C. Samples were collected at various times during this synchronized cycle and centrifuged, and the pellet was treated with saturated indole in MGT buffer (10^{-2} *M* Tris buffer, pH 7.5, containing 5×10^{-4} *M* $MgCl_2$). This treatment effectively breaks open the pellicle, and the cytoplasmic components with intact nuclei are obtained in suspension. Each sample added to the gradient volume consisted of about 20 ml of lysed cells. The gradient volume was 1000 ml, varying from 10 to 30 to 55% sucrose containing 10^{-2} *M* Tris buffer and 5×10^{-4} *M* Mg^{2+}. A 55% sucrose solution was added as a cushion, with 200 ml of Tris buffer added to push the sample inboard. The ribosomes were separated by centrifugation for 2–2½ hr at 40,000 rpm, reaching a total field force of $10.2 \times 10^{10} \omega^2 t$. Tracings were obtained by displacing the rotor contents with 55% sucrose through a Beckman DB spectrophotometer equipped with a 2-mm flow cell and through a Waters model 35 H refractometer.

Results and Discussion. – Zonal centrifugation of synchronized Tetrahymena taken at the end of the cold period shows substantial amounts of polyribosomes. Predivision warm-period cells show a decrease in single 82S ribosomes but no major changes in the higher multiple aggregates of polyribosomes. Cells treated with actinomycin D (10 μg/ml) 1 hr before the shift from the cold period to the warm period but lysed 1 hr after the onset of the warm period showed an increase in 82S single ribosomes and a very noticeable reduction in polyribosomes. Analysis by means of the Beckman model E ultracentrifuge further showed that anomalous 70S ribosomes are present only in actinomycin D–treated and postdivision cells. From all these findings it is suggested that a

breakdown of polyribosomes occurs in cells which are not actively engaged in protein synthesis and morphogenesis.

References

[1]Subcellular and Viral Physiology section.

[2]N. G. Anderson, H. P. Barringer, E. F. Babelay, and W. D. Fisher, *Life Sci.* **3**, 667 (1964).

[3]G. M. Padilla and I. L. Cameron, *J. Cellular Comp. Physiol.* **64**, 303 (1964).

1.5 CORRELATION OF EXCISION RATES WITH REPAIR OF PREMUTATIONAL LESIONS IN *ESCHERICHIA COLI*

Jane K. Setlow M. E. Boling

Introduction. – One approach to an understanding of the molecular basis of ultraviolet(uv)-induced mutation has been to determine the postirradiation conditions which maximize or minimize the mutational effects of uv. A study of repair of premutational lesions which is presumed to occur when the mutational effects are minimized could yield information on the mutation process itself. Therefore, an attempt has been made to correlate this type of repair under various conditions with rates of excision of thymine-containing dimers from the cellular DNA, a process which is believed to contribute to repair resulting in higher survival of irradiated cells.

Results and Discussion. – The most favorable condition for minimizing uv-induced revisions to prototrophy results in very poor excision. When nutrient-broth-grown cells emerging from the lag phase are irradiated and then placed in minimal medium plus glucose before plating, more than 80% of the potential mutations to prototrophy are lost,[1] a process called mutation frequency decline (MFD). However, irradiated cells emerging from the lag phase show poor excision rates regardless of the postirradiation medium. Excision is best when stationary or log-phase cells are irradiated and then placed in the same type of medium in which they were grown. The same cells grown in nutrient broth show a somewhat reduced excision rate in minimal medium plus glucose. Nutrient-broth-grown stationary or log-phase cells placed in minimal medium plus glucose show little MFD.

In spite of these data, three lines of evidence support the idea that excision is one step in the repair of premutational lesions reflected in MFD: (1) A mutant which is poor in MFD even under the most favorable conditions also has a slow excision rate under maximum excision conditions. (2) Glucose, as well as MFD, is apparently required for an appreciable rate of excision. (3) Acriflavine inhibits both processes. These conflicting lines of evidence may be reconciled if it is assumed that under conditions of maximum MFD the premutational lesions on a particular part of the bacterial DNA are especially susceptible to the excision enzyme, unlike those on the majority of the DNA, whereas measurement of excision rate reflects the repair of the DNA as a whole. It is concluded that excision of premutational lesions probably plays a role in minimizing the mutational effects of uv.

References

[1]E. M. Witkin, *Proc. Intern. Congr. Genet., 10th, Montreal, 1958* **1**, 280 (1959).

1.6 REPAIR OF ULTRAVIOLET DAMAGE IN *MICROCOCCUS RADIODURANS*

M. E. Boling Jane K. Setlow

Introduction. – *Micrococcus radiodurans* is extraordinarily resistant to ultraviolet (uv) as well as ionizing radiation. It has been postulated that this microorganism has a very efficient repair mechanism for thymine-containing pyrimidine dimers in the cell's DNA,[1] and it has been shown that these uv lesions are not responsible for most of the uv killing of the microorganism.[2] The fate of the thymine-containing dimers at various times after uv irradiation of cells labeled with tritiated thymidine has been followed for the purpose of determining the nature of the repair system of these dimers.

Results and Discussion. – Dimers are rapidly excised from the acid-insoluble cellular fraction after irradiation of log-phase cells. The rate of the reaction depends approximately inversely on the initial number of dimers formed in the cells (proportional to uv dose), as would be expected if this were a simple enzyme-substrate reaction

with an excess of substrate (the dimers in the DNA). A negligible fraction of the dimers which disappear from the acid-insoluble fraction of the cells appear in the soluble fraction. However, almost all the dimers lost from the cells are recoverable from the medium outside the cells. Increasing amounts of other radioactive material, not containing dimers, are also found in the medium with increasing time following uv irradiation, indicating that there is uv-induced breakdown of up to one-tenth of the DNA, in addition to elimination of dimers. Chromatography of the thymine- and thymine-dimer-containing material which is released into the medium by the irradiated, repairing cells indicates that the DNA fragments are not larger than trinucleotides and that probably most of the dimer-containing material does not contain a terminal phosphate, as judged by the mobility of the material on Whatman DE-20 paper before and after treatment with alkaline phosphatase.

It is concluded that *Micrococcus radiodurans* is resistant to uv partly because large numbers of pyrimidine dimers can be rapidly excised from the DNA and extruded into the medium. The pieces of dimer-containing material cut out of the DNA are probably relatively short.

References

[1]J. K. Setlow and D. E. Duggan, *Biochim. Biophys. Acta* **87**, 664 (1964).

[2]J. K. Setlow and M. E. Boling, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1964*, ORNL-3601, p. 34.

1.7 FURTHER PURIFICATION OF THE YEAST PHOTOREACTIVATING ENZYME

Amir Muhammed

Introduction. – A large-scale method for preparation of purified photoreactivating enzyme from commercial bakers' yeast has been developed. The enzyme has been assayed by its ability to increase transformation by ultraviolet-irradiated *Hemophilus influenzae* DNA in the presence of 3000- to 4000-A radiation.

Results and Discussion. – The method consists of drying 50 lb of fresh yeast and resuspending the dried material in phosphate buffer. The resulting crude extract is centrifuged, and about 22 liters of supernatant is collected. The enzyme is then purified from the supernatant by ammonium sulfate precipitation, Sephadex G-25 filtration, calcium phosphate gel adsorption, and DEAE-cellulose, phosphocellulose, and hydroxylapatite column chromatography.

The specific activity of the final preparation is approximately 3000-fold greater than that of the crude extract. The overall yield of enzyme is about 6%. Analytical centrifugation of a concentrated final preparation indicates the presence of one large, homogeneous component of molecular weight about 30,000 and another small component which does not show a peak in Schlieren pattern but absorbs light at 546 mμ. Preliminary analytical electrophoresis of the purified enzyme on acrylamide gel shows most of the protein moving towards the cathode at pH 7.0 as a single band, with a faint smear in the other direction, suggesting that the enzyme is positively charged.

Determination of the nature of the light-absorbing part of the enzyme depends on further purification. At present it is not possible to state whether the 546-mμ absorbing material is a contaminant or part of the enzyme.

1.8 ATTEMPTED SUICIDE WITH ^{33}P IN BACTERIOPHAGE T4

C. M. Steinberg Diane J. Goins[1]

When ^{32}P is incorporated into the DNA of viruses and bacteria, the decay of this radioactive isotope results in the death of the organism with an efficiency of about 0.1 per disintegration. It has been demonstrated that this is the result of the transmutation process itself, that is, it is not due to the beta radiation associated with the decay. The principal interest in this phenomenon lies in the desire to understand the reasons for the efficiency factor; naively, one could have expected that the consequences of transmutation should either be lethal or nonlethal. Various theories have been proposed to account for the observed efficiency; the most reasonable of these postulates is that killing is caused by the recoil of the ^{32}S which often breaks its own polynucleotide strand and breaks the opposite strand with 0.1 efficiency. This hypothesis predicts that a phosphorus isotope emitting a lower-energy beta particle would have a drastically lowered killing efficiency.

In addition to ^{32}P, which has a beta radiation energy of 1.71 Mev and a half-life of 14 days, there is another isotope of phosphorus, ^{33}P, which has a beta energy of 0.25 Mev and a half-life of 26 days. It has been shown that it is feasible to produce this isotope from sulfur enriched in ^{33}S by exposure to thermal neutrons.[2] By this method, 0.6 mc of ^{33}P was produced in 50 mg of sulfur enriched to 68.1% in ^{33}S.[3] Also, it has been shown that it is possible to produce millicurie amounts of ^{33}P in the reaction $^{36}Cl(n,\alpha)^{33}P$.[4]

Phosphorus-33 produced by both these processes was used in suicide experiments with the bacteriophage T4. It was possible to demonstrate that the killing efficiency is less than 0.02 for ^{33}P incorporated in the bacteriophage.

References

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[3]C. M. Steinberg, R. C. von Borstel, H. R. Gwinn, E. W. McDaniel, S. A. Reynolds, and R. E. Lewis, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1965,* ORNL-3768, p. 40.

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1.9 VARIATION IN RATE OF DNA SYNTHESIS DURING S PERIOD IN SYNCHRONIZED MAMMALIAN CELLS

E. H. Y. Chu Lana T. Covington
Margaret R. Kasschau

Introduction. – An improved method has been developed to obtain large quantities of highly synchronized Chinese hamster cells in vitro. In the absence of any chemical agent to synchronize the mitotic cycle, it was possible, under almost unaltered physiological conditions, to (1) estimate the duration of the DNA-synthetic (S) period, (2) demonstrate variation in rate of synthesis during various stages of S, and (3) compare the efficacy of the autoradiographic and liquid scintillation counting procedures for determining the incorporation of radioactive precursors.

Methods. – Cells were grown in monolayers in Roux flasks for 18–20 hr. After pulse labeling for 30 min with ^{3}H-thymidine, the cells were placed in a calcium-deficient medium. The cultures were stored at 4°C for 1 hr before reincubating them at 37°C. As cells approach mitosis, they usually round up, become tenuously attached to the substrate, and can be selectively dislodged by agitation.[1,2] From the same culture, successive samples were collected in this manner at every hour, after which the cultures were reincubated in fresh calcium-deficient medium. Part of each sample was used for the determinations of cell number, mitotic index, and labeling index (percent of labeled cells and number of silver grains per cell); the remaining was used for measuring the total incorporated radioactivity in the acid-insoluble material per cell by the liquid scintillation counting method.[3]

Results and Discussion. – From 1 to 1.5% of the original inoculum was harvested by agitation at each hour. Ninety-nine percent of cells collected were viable, and over 90% of these were in mitosis. In separate experiments, these cells, when reincubated, entered the pre-DNA-synthetic period (G_1) and synchronously completed the cell cycle. The cell number doubled in 17 hr.

At the time of pulse labeling in the present studies, cells in all phases of S in the previous cycle incorporated the radioactive precursor. As these cells successively entered mitosis, they selectively were collected. Thus, each periodically collected sample consisted of a population of cells which at the time of labeling were in some stage of S, and the measure of incorporated radioactivity in each hourly sample represents the rate of DNA synthesis at the particular phase of S. Our results from the two methods agreed very well, and each method was reproducible.

Cells collected during the first 4 hr in 37°C after chilling were in the post-DNA-synthetic period (G_2). The duration of G_2 was prolonged by about 1 hr, possibly because of chilling. The duration of S was 8 hr, confirming our earlier result in this cell line.[4] The rate of synthesis rose rapidly and steadily, reaching a peak at the fifth hour of S; it became somewhat slower during the remaining 3 hr. These results confirm the data of Dendy and Cleaver[5] on mouse L cells, which show that the rate of DNA synthesis during S is variable but differ in details on rates in different parts of S, possibly because different cells and methods were employed. By comparing the present data with

those[6] obtained in 5-aminouracil–synchronized populations of Chinese hamster cells, it appears that the chemical probably blocked cells at the beginning of S and did not significantly modify the rate of DNA synthesis in the treated cells.

References

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1.10 NUCLEIC ACID SYNTHESIS IN HUMAN CELLS IN SWIRL CULTURES

J. D. Regan[1]

Patterns of DNA and RNA synthesis have been studied in 5-aminouracil–synchronized human cells (strain HCAAT) in swirl cultures. Experimental cultures were treated with 5-aminouracil (5AU) for 24 hr. The cells were then centrifuged, and both control (untreated) and 5AU-treated cultures were resuspended in fresh medium. The time of 5AU removal was referred to as time zero. The synthesis of DNA and RNA was measured by the amount of incorporation of tritiated precursors into cold-acid-insoluble material after periodic (usually hourly) pulse labels.[2]

Figure 1.10.1 shows DNA and RNA synthesis in control and 5AU-synchronized cells. In the control culture, previous to medium change, both DNA synthesis and RNA synthesis proceeded at rates typical for pre- and early-log-phase populations of this cell line. At time zero, when the cells were centrifuged and placed in fresh medium, incorporation of both ^{3}H-thymidine and ^{3}H-uridine was minimal. The synthetic activities remained at a low level for about 15 hr before moving up toward the log-phase rate.

In the presence of 5AU, both DNA synthesis and RNA synthesis were at minimal levels. When the 5AU was removed, there was a burst of DNA synthesis which began immediately and continued to increase, reaching a peak at about 5 hr. This activity decreased rather sharply, reaching minimal

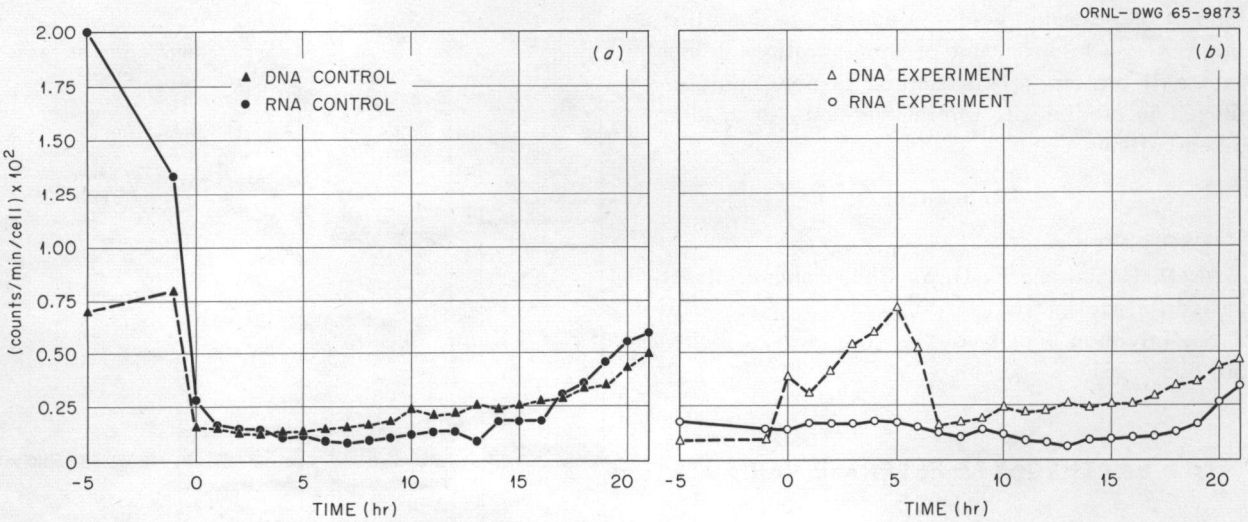

Fig. 1.10.1. DNA and RNA Synthesis in HCAAT Cells. Cell density at time zero was 5 to 6 × 10^5 cells/ml in control and experimental cultures. (*a*) Control. At time zero, cells were placed in fresh medium. Incorporation of both precursors was minimal for 15 hr and then gradually increased. (*b*) 5AU treated. 5AU removed at time zero. No ^{3}H-uridine incorporation occurred during or after first DNA synthesis.

levels again at about 7 hr. In several repeated experiments, RNA synthesis always remained at minimal levels during the first DNA synthesis after 5AU treatment. In experiments of 30 hr duration, RNA synthesis did occur in the second S period; incorporation of ^{3}H-uridine followed the pattern of ^{3}H-thymidine incorporation but lagged behind about 1 hr.

Failure to incorporate ^{3}H-uridine during the first S period may be due to the 5AU treatment itself, possibly by a flooding of the uridine pool with the analog. The primary effect of 5AU in mammalian cells is not known. It is clear, however, that both DNA synthesis and RNA synthesis are minimal during the 5AU treatment and that the DNA-synthetic system recovers immediately upon removal of the analog. If it is assumed that the same is true for RNA synthesis, then it is possible that replication of the DNA of the inhibited cell is necessary first, before any transcription of messenger and other RNA can occur.

The pattern of protein synthesis, as measured by incorporation of ^{3}H-amino acids in 5AU-synchronized cells, is under study. In routine medium it is difficult, however, to get much label into cell protein in even an extended pulse label. This is probably due to the large cell pool of amino acids[3] and to the amino acids in the medium. In recent experiments, cells were grown on medium deficient for certain essential amino acids and were then placed in medium which contained the tritiated form of these amino acids. Under these conditions there was a high amount of incorporation of label into cell protein. This method is now being employed in studies of protein synthesis in synchronized cells.

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1.11 ERYTHROCYTE ESTERASE VARIANTS IN MICE

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

Introduction. – Zymograms of lysates of washed erythrocytes of inbred strains of mice show three patterns of acetylesterases following starch gel electrophoresis. Tests were made to determine the inheritance of these red-cell esterases.

Materials and Methods. – Blood from several inbred strains of mice was collected in 2% sodium citrate to prevent clotting. Red cells were separated by centrifugation and washed three times in ten volumes of 0.85% saline. Washed red cells were pipetted onto pieces of Whatman No. 1 filter paper used for the paper insertion technique in starch gels[1] prepared in 0.03 *M* borate buffer at pH 8.5. Lysis occurred after the paper insert was placed in the gel. Horizontal electrophoresis at room temperature at 5 ma and a voltage drop of 7.5 v/cm was stopped after 4 hr. The erythrocyte esterases were located by histochemical methods,[2] using α-naphthyl acetate as substrate and Fast Blue RR Salt as the diazonium dye complex.

Results and Discussion. – The three esterase patterns are shown in Fig. 1.11.1. The esterase phenotype Ee-1a is illustrated by strain C57BL mice, Ee-1b by strain C3H mice, and Ee-1c by strain RFM mice. Pelzer[3] has observed Ee-1a and Ee-1b and has shown that they are controlled by alleles at a single autosomal locus; he did not observe Ee-1c among the 11 inbred strains studied. Offspring of mice of Ee-1c and Ee-1a or Ee-1b phenotypes have both types of esterases, each in

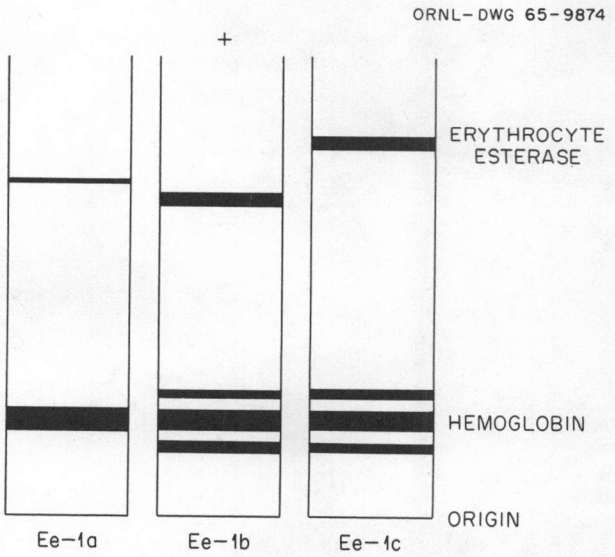

Fig. 1.11.1. Erythrocyte Esterase Variants in C57BL, C3H, and RFM Mice.

Table 1.11.1. Backcross and F_2 Progeny Classified for Erythrocyte Esterase Genotypes

Mating	Offspring			Total
	$\frac{Ee\text{-}1^b}{Ee\text{-}1^b}$	$\frac{Ee\text{-}1^b}{Ee\text{-}1^c}$	$\frac{Ee\text{-}1^c}{Ee\text{-}1^c}$	
$\frac{Ee\text{-}1^b}{Ee\text{-}1^c} \times \frac{Ee\text{-}1^b}{Ee\text{-}1^b}$	40	37		77
$\frac{Ee\text{-}1^b}{Ee\text{-}1^c} \times \frac{Ee\text{-}1^b}{Ee\text{-}1^c}$	6	11	5	22

reduced amounts. The esterase phenotypes of some of the backcross and F_2 progeny examined are given in Table 1.11.1. The ratios suggest that the Ee-1c phenotype is controlled by an allele at *Ee-1*. The suggested symbol for the allele in strain RFM mice is $Ee\text{-}1^c$.

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1.12 POSITION OF AMINO ACID REPLACEMENTS IN THE α CHAIN OF MOUSE HEMOGLOBIN

R. A. Popp

Introduction. – Differences in the solubility and crystalline precipitates of mouse hemoglobins in K_2HPO_4-KH_2PO_4 buffers are associated with modifications within the α chain.[1] Fingerprints of the tryptic peptides suggest that one or two of the peptides have amino acid replacements. The purpose of this study was to determine what amino acid replacements do occur and to locate their positions within the α-chain polypeptide.

Methods and Results. – The tryptic peptides of the α chain of hemoglobin from C57BL, BALB/c, and NB mice were isolated by column chromatography over Dowex 50-X2 and Dowex 1-X2 resins.[2] Amino acid analyses revealed that the amino acid compositions of peptides αT-4 of NB and αT-9 of NB and BALB/c differed from the amino acid compositions of the corresponding peptides of C57BL hemoglobin (Table 1.12.1). The tryptic peptides were partially degraded by other enzymes, such as pepsin and chymotrypsin, to obtain small peptides suitable for complete sequence studies. Edman degradation procedures[3] were used to determine the sequence of amino acids in these peptides. The results are compared with the C57BL α chain as a standard reference. The position of the Val substitution for Gly in αT-4 of NB is presently under investigation. The replacement

Table 1.12.1. Tryptic Peptides in the α Chain of Hemoglobins from C57BL, NB, and BALB/c Mice That Show Amino Acid Replacements

Strain	Peptide	Amino Acid Residues														
		Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ileu	Leu	Tyr	Phe
C57BL	αT-4		1	1				3		4	3		1	1	1	
NB	αT-4		1	1				3		3	3	1	1	1	1	
C57BL	αT-9	1	3		5		2		1	2	8	1		6		
NB	αT-9	1	3		4		3		1	2	8		1	6		
BALB/c	αT-9	1	3		4	0.5	2.5		1	2	8	1		6		

of Val by Ileu in αT-9 of NB occurs in position 62. In position 68 of C57BL hemoglobin, Asn is replaced by Ser in αT-9 of NB hemoglobin, but it is replaced by ½ unit of Ser and ½ unit of Thr in BALB/c hemoglobin.

Discussion and Conclusion. – The solubility and crystalline properties of NB hemoglobin are more similar to those of C57BL than to BALB/c hemoglobin.[4] However, NB hemoglobin has a larger number of amino acid replacements than does BALB/c hemoglobin (Table 1.12.1). We previously reported[5] an Asp to Thr replacement in BALB/c αT-9. However, a more thorough study showed that the Asp residue in C57BL hemoglobin is Asn, and peptide αT-9 of BALB/c hemoglobin contains ½ unit of Thr and 2½ units, rather than 2 units, of Ser. These results could be obtained by either of two methods. The nucleotide triplet (codon) may be ambiguous such that it codes for both amino acids or, more probably, the α-chain locus is compound and is composed of two cistrons, and an independent mutation has occurred in one of them. Genetic and chemical studies are in progress to determine which of these is correct.

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1.13 SPLEEN WEIGHT AS A MEASURE OF MARROW CELL GROWTH IN IRRADIATED MICE

R. A. Popp C. C Congdon[1]
Joan Wright Goodman[1]

Introduction. – Bone marrow cells that are infused into irradiated mice proliferate in the spleen as well as in the marrow cavities. Observations on the recovery of spleen weight following x irradiation and marrow treatment[2] suggest that spleen weight might be used to assess the growth of transplanted marrow cells in mice. Thus, spleen weights were compared with the number of spleen nodules[3] or the percentages of ^{59}Fe uptake[4] in the spleens of marrow recipients to determine whether data obtained by each assay system correlated with one another.

Materials and Methods. – (C57BL × C3H)F_1 or (C57BL/6 × DBA/2)F_1 mice received a lethal dose of x rays followed by an intravenous injection of a measured number of parental bone marrow cells. Mice used for comparative spleen weight and ^{59}Fe-uptake assays received ~0.5 μc of ^{59}Fe 24 hr before being killed. Mice were killed between 4 and 21 days after bone marrow treatment for spleen weight assays. Spleen colony and spleen weight data at 10 days were compared, and ^{59}Fe uptake and spleen weight data at 4 and 10 days were compared.

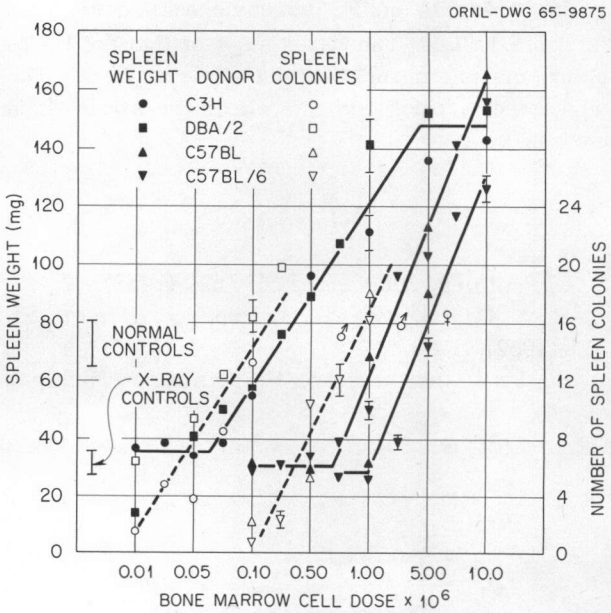

Fig. 1.13.1. Spleen Weight and Spleen Colony Response in Lethally Irradiated BC3F_1 and B6D2F_1 Recipients of 10^4 to 10^7 Parental Marrow Cells. All mice were killed 10 days after treatment. Each point represents an average from 10 or more mice; standard errors of the mean are shown for a few of the points. Male and female recipients of DBA/2 and C3H parental marrow showed similar spleen weight and spleen colony response, but the values for male recipients of C57BL or C57BL/6 parental marrow were greater than for females. The solid symbols and solid lines represent spleen weight, and the open symbols and broken lines represent spleen colony.

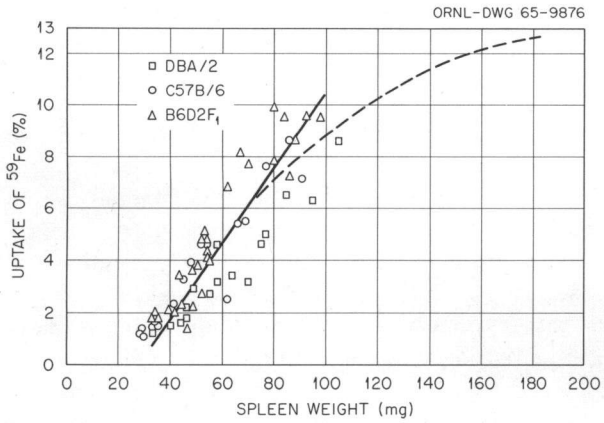

Fig. 1.13.2. Spleen Weight and ^{59}Fe Uptake in Lethally Irradiated B6D2F$_1$ Recipients of Isologous or Parental Marrow Cells. Irradiated mice received an inoculum of 3.2×10^4 to 3.2×10^7 marrow cells on day 0. Approximately 0.5 μc of ^{59}Fe was administered intraperitoneally on day 5, and the mice were killed on day 6. Each point represents an individual mouse killed on day 6. The broken line represents an average of the 24-hr ^{59}Fe uptake values obtained from individuals sacrificed on days 8 and 10 after treatment (the individual points are not shown for reasons of clarity).

Results and Discussion. – The donor-recipient combinations studied showed a linear relation, within the defined limits, between cell dose and spleen weight and spleen colony formation (Fig. 1.13.1). Spleens with fewer than five colonies did not show a significant increase in weight above that of irradiation controls. Spleen weight assays were particularly useful for large cell inocula that resulted in confluence of colonies. Spleen weight and ^{59}Fe uptake showed a linear relationship for individuals with spleens that weighed less than 100 mg, but ^{59}Fe uptake became less than proportional to spleen weight in spleens that weighed more than 100 mg (Fig. 1.13.2).

The results suggest that spleen weight, spleen colony formation, and ^{59}Fe uptake are equally suitable assays, within appropriate limits, for studies on proliferation of marrow cell transplants in irradiated mice. Presumably this is because the weight of the spleen is a reflection of the number of cells in the spleen of lethally irradiated recipients which arise from colony-forming cells in the marrow inoculum to produce clones of cells that incorporate ^{59}Fe.

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1.14 POLYTENE CHROMOSOME METABOLISM

Crodowaldo Pavan

Our work in the first five months at ORNL was related to the maintenance of *Rhynchosciara angelae* in our new laboratory, and in doing some experiments on the normal and abnormal metabolism on the polytene chromosomes of this fly.

Normal Metabolism. – We are doing a comparative study on the synthesis of nucleic acids and protein in three different tissues: the salivary gland, the Malpighian tubules, and the median intestine. As we already had some data about the activities of the polytene chromosome in these tissues at different stages of larval development,[1,2] we were interested to know how much the morphological transformations observed reflect the chemical changes expected. On the other hand, since many of the chemical reactions which go on in the chromosomes are not followed by visible morphological changes, we were interested to analyze and compare the corresponding changes in the three tissues. We are using ^{3}H-thymidine, ^{3}H-cytidine, and ^{3}H-uridine for the analysis of the synthesis of nucleic acids, and ^{3}H-leucine, ^{3}H-lysine, and ^{3}H-phenylalanine for the synthesis of protein.

Our preliminary results show that in relation to all these radioactive precursors, the activity of the cells of the salivary gland is much higher than that of the other tissues.

Independent of the tissue considered, however, when a chromosome shows a puff there is always an intense synthesis of RNA in this locus. The rate of synthesis as well as of turnover of RNA is different for different tissues. Even in the same organ, for example, the Malpighian tubules, cells from different regions show evident differences in the pattern and rate of synthesis of RNA.

It is interesting to notice that, although the cells of practically all tissues show many times a

higher incorporation of RNA precursors at the nucleolar-organizing region, no evident nucleolus is normally observed in these cells. It appears that, in the tissues we studied, many times the cells have diffused nucleoli represented by a great number of micronucleoli. As granules of the size and appearance of these micronucleoli are observed inside many puffs, we think that they can be produced in many different loci along the chromosomes, and not only by the nucleolus-organizing region. In many individuals of a group of larvae which show some abnormality in development, we found cells of the three studied tissues showing typical nucleoli at the base of X chromosome where the nucleolus-organizing region is localized. The cells of these larvae, when maintained for about 1 hr in Ringer's solution specially prepared for *R. angelae*, showed nucleoli much larger than those seen in the solution prepared soon after the sacrifice of the animal.[3]

As for the metabolism of DNA, it appears that, although the basic pattern of synthesis is very similar even if we consider chromosomes of different organs, there is a great difference in the rate of synthesis when we consider cells of different tissues as well as when we consider different regions of the same chromosomes. In this last case the great differences are observed in loci which show production of metabolic DNA in one tissue and not in others.

The results we are obtaining about the synthesis of protein are very inconclusive. It appears that the precursors which we are using now are in some way different from the one we used anteriorly.[4] Although having the same chemical designation (produced by different firms), they gave very different results.

Part of the results here summarized will be published in the *Proceedings of the Brookhaven Symposium in Biology*, 1965.

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1.15 METABOLISM IN HYPERTROPHIED CHROMOSOMES

Crodowaldo Pavan

Abnormal Metabolism. – Recently we found two microorganism infections in cells of the salivary gland and of the gastric ceca of *R. angelae*, which produced changes in their polytene chromosomes.[1] The infection in the cells of the salivary gland possibly due to Microsporidia produced an increase in the size of the cells and their chromosomes. The infection affected not only the size of the chromosomes but induced also the formation of a new type of puff (puff in the heterochromatin) in the heterochromatic regions of all chromosomes. Structurally, these puffs are a combination of a nucleolus and a large puff in a band of the polytene chromosome. The infection in the cells of the gastric ceca was due to a DNA virus, which induced a large increase in the size of the cell and of its chromosomes, as well as the production of many polyhedral crystals by the cell. In this infection, however, there are no special changes in the heterochromatic regions, but the chromosomes show change in the banding pattern represented mainly by the appearance of light regions in some chromosomal loci. We still don't know the exact meaning of these light regions; they may be a special kind of puff of a gap in one in a few DNA bands.

Another evident change induced the fragmentation of the chromosomes, which is very frequently observed in heavily infected cells.

In our current experiments, in addition to those concerning the abnormality in nucleoli formation already mentioned, we find cases of changes in chromosome sizes, in the pattern of puff formation, and in the heterochromatic regions. We still cannot determine the cause of the induced changes observed in our last experiments, but we have good indications that many pathological conditions may act as inductors of changes in the chromosome morphology and behavior.

As in polytene chromosomes, we may study the reaction of the individual genes to different pathological conditions. We hope they will help us to understand the normal and abnormal metabolism of diploid cells.

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1.16 SPERMATOGENESIS IN *RHYNCHOSCIARA ANGELAE*

Renato Basile

R. angelae is a large member of the Sciaridae that presents some peculiar cytological characteristics during the process of formation of the sexual cells. Many of these characteristics are present also in *Sciara coprophila*, which was studied by Metz and collaborators about 30 years ago, and which has some important implications in recent work done on the behavior of the chromosomes of mammals.

We followed the spermatogenetic process from the very young larvae to the adult stage of *R. angelae*. This is facilitated by the biological pecularities of this fly, which shows a synchronous development of all larvae (from 1500 to 2000) produced by a single female. It is interesting to notice that *R. angelae* shows this synchronous behavior in all larvae of a group, and also a synchrony in the behavior of practically all cells of the gonad. At any time of development of the gonad, all the cells are in the same stage of development. This makes *R. angelae* an excellent material to study the effects of radiation and other physical and chemical agents in the production of mutation at different stages of development of the sexual cells.

The meiosis in this fly is similar but not equal to the one observed by Metz and co-workers in *S. coprophila*. In *R. angelae*, as in *S. coprophila*, one spermatocyte will give origin to a single spermatozoon and not four as is normally formed in other organisms. During spermatogenesis there is preferential elimination of chromosomes, indicating the existence of chromosomal differentiation, a problem which we are very much interested to study.

The description of spermatogenesis in *R. angelae* and a comparison with what happens in *C. coprophila* is being prepared and will be published soon.

1.17 OOGENESIS IN *RHYNCHOSCIARA ANGELAE*

Renato Basile

The ovary of *R. angelae* is formed by many follicles. Each follicle has three types of cells: the oocyte, the nurse cell, and a great number of little follicular cells. In the young larvae, the ooctyes are very small and show the chromosomes in synapsis. The heterochromatic regions are fused in one chromocenter, from which arise five long arms of paired chromosomes. The young nurse cell (there is only one nurse cell for oocyte) has ten chromosomes: three pairs of autosomes, one pair of X chromosomes, and one pair of limited chromosomes. The limited chromosomes remain condensed, while the others show special aspects, indicating that they are very active. They present the heterochromatic region surrounded by loops of euchromatin. Their appearance reminds us in some ways of the lampbrush chromosomes of the amphibians.

From the young larva stage until pupation the ovary shows little increase in its volume, but during pupation the increase is such that in the adult fly the ovary occupies about 80% of the abdomen volume. During the development of the ovary, a process of polyploidy associated to a polyteny is shown to occur in the chromosomes of the nurse cells. The polyploidy begins when the larva is about 35 days old and continues until the middle of the pupa stage, the number of chromosomes increasing from 10 to approximately 300. The limited chromosomes do not follow this endomitotic process, remaining condensed.

During the middle of the pupation stage, the endomitosis stops, and the chromosomes which were in a loosely polytene stage show an intimate pairing of the chromonemata and a typical aspect of polytene chromosomes. The oocyte itself, which until this moment showed practically no increase, now increases enormously, and the ovary shows a rapid and great increase. We are very much interested in studying the relation between the increase of the oocyte and the material produced in the nurse cells and transferred to it.

We already have some evidences, using the autoradiographic method and radioactive precursors of nucleic acids, that, from the nurse cells and probably from the follicular cells, RNA is transferred to the oocyte.

We are now analyzing the synthesis of DNA, RNA, and proteins in the nurse cells and oocyte, and we are studying the process of transfers of some of those substances from one cell to others.

Chromosome Cytology

Sheldon Wolff
M. Ellen Mattingly
Sandra L. Bell[a]
J. A. Heddle[b,c]
W. J. Peacock[a]
J. E. Trosko[d]
H. E. Luippold

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[d]American Cancer Society Fellow.

2.1 DOSE INDEPENDENCE OF TRITIATED THYMIDINE–INDUCED CHROMOSOME ABERRATIONS IN *VICIA FABA*

Sheldon Wolff H. E. Luippold

Although it has long been known that the endogenous radiation from tritiated thymidine can break chromosomes, it has usually been thought that sister-strand exchanges found in tritiated thymidine–treated material represent spontaneous sister-strand crossovers. One of the reasons for thinking this is that sister-strand exchanges are independent of dose over the range of concentrations of ^{3}H-thymidine tested.

When lateral roots of *Vicia faba* were treated for 4 hr with various concentrations of ^{3}H-thymidine (specific activity of 2.0 curies/millimole) or with various activities of ^{3}H-thymidine (specific activity varied, total thymidine concentration kept constant), it was found that the isochromatid aberrations induced by the endogenous radiation first rose with increasing dose but soon reached a plateau between 5 μc/ml and 10 μc/ml. Cells in roots treated with 5 μc/ml of ^{3}H-thymidine had fewer grains in autoradiographs than did cells treated with 10 μc/ml. The numbers of grains were not decreased by a factor of 2, however, showing that there was some saturation of incorporation of radioactive precursors also.

The independence of aberration yield and dose of ^{3}H-thymidine lends support to the hypothesis that the sister-strand exchanges seen in autoradiographs are not spontaneous sister-strand crossovers but merely exchanges induced by the radiation from the ^{3}H-thymidine.

2.2 STUDIES OF THE EFFECTS OF 5-FLUOROURACIL DEOXYRIBOSIDE ON *VICIA FABA*

Sandra L. Bell Sheldon Wolff

5-Fluorouracil deoxyriboside (FUdR) has been shown to inhibit DNA synthesis in several organisms including *Vicia faba*.[1–3] This inhibition is reversed by the addition of exogenous thymidine.

In autoradiographic studies in *Vicia* it has been shown that in the presence of equal concentrations of FUdR and thymidine there is no effect of FUdR on ^{3}H-thymidine incorporation.[4] This would indicate that a concentration of thymidine equal to the concentration of FUdR will reverse the inhibition of DNA synthesis. In the presence of equal concentrations of FUdR and thymidine, however, ^{3}H-deoxycytidine incorporation is completely inhibited. Even at very high concentrations of thymidine (100 and 1000 times higher than the FUdR concentration), the incorporation of ^{3}H-deoxycytidine never reaches the level of the controls. Two explanations for these results were previously proposed.[5] These explanations were that (1) deoxycytidine inhibits DNA synthesis in the presence of FUdR, and (2) FUdR stimulates the production of endogenous deoxycytidine so that the exogenous ^{3}H-deoxycytidine is a smaller percentage of the pool of deoxycytidine present for DNA synthesis in the FUdR-treated roots than in the control roots.

In experiments performed to test the first of these hypotheses, lateral roots were grown in 10^{-5} *M* FUdR, 10^{-5} *M* deoxycytidine, and ^{3}H-thymidine. Grain counts on autoradiographs indicated that deoxycytidine in either the presence or the absence of FUdR had no effect on ^{3}H-thymidine incorporation.

In preliminary experiments to test the second hypothesis, the acid-soluble nucleotides of roots grown in 10^{-5} *M* FUdR and various concentrations of thymidine were separated on an ion exchange column using Dowex 1-X8, as previously described by Burger *et al.*[6] An acetate gradient (pH 4.4) ranging from 0.15 to 3 *M* was used to elute the nucleosides and nucleotides. It was not possible to separate the deoxynucleoside from the equivalent riboside and bases by using this method. Although there was a definite depression in the deoxycytidine-cytidine-cytosine peak in the presence of FUdR and high concentrations of thymidine, there were also effects on other peaks which make these results difficult to interpret.

Of our two possible hypotheses to explain the effects of FUdR and thymidine on ^{3}H-deoxycytidine incorporation in roots of *Vicia*, we have disproved one hypothesis and have very tentative data in support of the other.

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2.3 MITOTIC INHIBITION BY RNA-SYNTHESIS INHIBITORS

M. Ellen Mattingly

Although numerous investigators have studied the inhibitory effects of actinomycin D and 8-azaguanine on RNA synthesis,[1] relatively little is known of the relation of such inhibition to the decrease in mitotic index observed in higher organisms. This problem has been studied in *Vicia faba*.

In recent experiments, lateral roots of *Vicia* were exposed continuously to either actinomycin D (50 μg/cc) or 8-azaguanine (5×10^{-4} *M*), and at intervals up to 30 hr, roots were fixed, stained, and smeared. The data in Table 2.3.1 show that in *Vicia* at the concentration of actinomycin D used the mitotic index remains high for 12 hr but then falls rapidly so that at 16 hr it reaches 3%; by the end of 30 hr, no divisions are seen. 8-Azaguanine, however, rapidly induces a severe inhibition of cell division so that few metaphase cells are seen even at the end of 12 hr.

Some information about the stage of the cell cycle affected by the two inhibitors was obtained by the use of ^{3}H-thymidine and autoradiography. Lateral roots were exposed to ^{3}H-thymidine (2 μc/cc; 1.9 curies/millimole) for 30 min, were rinsed, and then placed in either actinomycin D or 8-azaguanine. Table 2.3.1 shows that in the presence of 8-azaguanine some cells that were in the DNA-synthetic period (S) at the beginning of treatment were able to begin cell division and thus

Table 2.3.1. Effect of RNA-Synthesis Inhibitors on Mitotic Index

Hours After Beginning of Treatment	Mitotic Index			Percent Labeled Metaphases	
	Control	Actinomycin D	8-Azaguanine	Actinomycin D	8-Azaguanine
0	13	12	12	0	0
6	10	7	9	0	45
12	10	13	1	71	0
16	10	3	0	45	0
24	10	2	0	22	0
30	14	0	0	0	0

to appear as labeled metaphase cells at 6 hr, which is approximately the time at which labeled metaphase cells begin to appear in control roots. It seems, therefore, on the basis of these preliminary data that the stage in the cell cycle which is most sensitive with respect to mitotic inhibition in the presence of 8-azaguanine is some portion of S.

In the presence of actinomycin D, cells that were in S at the beginning of treatment seemed to be delayed in their entrance into mitosis, so that at the end of 6 hr in actinomycin D no labeled metaphase cells were seen. This delay could account for the slight drop in mitotic index seen at 6 hr. On the other hand, S cells did ultimately enter mitosis. Since after 12 hr (the time of maximum number of labeled metaphases) the mitotic index dropped rapidly, it is impossible to determine if all the S cells actually passed through mitosis. It is fairly certain, nevertheless, that few G_1 (pre-DNA-synthetic period) cells divided in the presence of actinomycin D.

The preliminary experiments are comparable to similar studies on the effects of actinomycin D on synchronous division in ciliates,[2] which have shown that there is a critical period in the interphase portion of the cell cycle with respect to inhibition of mitosis. Cells exposed to actinomycin D before this critical point will not divide; cells exposed after this point will divide once. The data reported here indicate that this critical point is associated with some aspect of the S phase. Experiments are now in progress to determine the effects of actinomycin D and 8-azaguanine on DNA synthesis.

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2.4 RANDOMNESS IN THE FORMATION OF RADIATION-INDUCED CHROMOSOME ABERRATIONS

John A. Heddle

Introduction. – The formation of radiation-induced chromosome exchanges involves two distinct steps, the breakage of the two strands and the subsequent reunion in a new configuration. Breakage is usually assumed to be random along the interphase chromosome, while rejoining to form an exchange is nonrandom and occurs only at sites at which the two strands are close together. If the broken ends rejoin at random within a site, the two centric pieces will join to form a dicentric with the same frequency that an acentric end and a centric end rejoin to form a translocation. In the usual cytological experiments the dicentrics are scored and the translocations are not, while in the usual genetic experiments only the translocations are scored. Thus, the relative frequencies of these two types were not determined, and neither could be known to give a true estimate of the radiation-induced changes that occurred.

Cytological experiments in which chromatid aberrations are scored, while permitting the recognition of both dicentrics and translocations, give conflicting results[1,2] and are subject to systematic scoring errors. I have been able to use chromosome rather than chromatid aberrations to determine the ratio of dicentrics to translocations involving an acrocentric chromosome and the region between the centromere and the secondary constriction of the large metacentric chromosome in *Vicia faba*. This particular translocation can be recognized cytologically regardless of the length of chromosomal material exchanged.

Materials and Methods. – Dry seeds were irradiated at 1000 r/min to give total doses of 5 or 6 kr. Seeds were germinated immediately after irradiation, grown for about two days, treated with 0.2% colchicine to accumulate metaphases, and then fixed in Ford's fixative at the time of the first mitotic divisions in the primary root. One slide was made from each root, and all the scorable metaphases were used.

Results. – The numbers of dicentrics and translocations are shown in the following table.

Dose	Approximate Number of Cells	Dicentrics	Translocations
5 kr	500	26	29
6 kr	100	8	4
Totals	600	34	33

It may be seen that these two aberration types occur with equal frequency.

Discussion. – The usual assumption made about dicentrics and translocations is that they represent two different patterns of reunion of the same initial breakage event. This assumption is made in spite of reports that for chromatid aberrations the two patterns are not found with equal frequency, dicentrics predominating in some experiments[2] and translocations in others.[1] By means of the method outlined above it was possible to score cytologically all translocations at the first mitotic division after irradiation when, presumably, there is no selection against any type of chromosome aberration. Furthermore, there is no error in scoring introduced by (1) anaphase movement, (2) failure in sister-chromatid pairing, or (3) inability to recognize translocations of nearly equal lengths of chromosomal material, all of which can lead to systematic errors. The results show that dicentrics and translocations arise with equal frequency and indicate that rejoining of broken ends is random within sites.

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2.5 INSECT TISSUE CULTURE

J. E. Trosko

It has been reported that embryonic cells isolated from *Drosophila* eggs can be cultured continuously.[1] Since this system potentially affords ways to approach many genetic and cytogenetic problems, attempts were made to exploit the system. It turns out, however, that these "tissue culture" cells are quite unlike normal mammalian cells in culture in that the rate of proliferation is very low. Although lines of cells have been maintained for six months, no cell attachment or significant cell growth has been achieved. Many attempts to improve the medium failed to raise the division rate. Cells were derived from sterile eggs and from larvae, pupae, and adults that were grown aseptically. Also, other lines of cells were derived from specific organs, that is, brains and testes of larvae, by dispersing the cells of the organ by trypsin-EDTA solution. Similar results were obtained when cells of *Rhynchosciara* and haploid *Habrobracon juglandis* were put into culture.

Rates of DNA synthesis of these cells were very low, as was indicated by the very small amount of ^{3}H-thymidine incorporation over a 24-hr labeling period. It appears quite obvious that this insect tissue system can be of value only if the rate of cell division can be increased, and therein lies the problem of finding the proper medium and conditions for insect cells.

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Fungal Genetics

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3.1 DOSE-RATE EFFECTS ON SURVIVAL AND MUTATION INDUCTION IN *NEUROSPORA* CONIDIA

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

Previous studies[1] on the genetic effects of high-intensity x irradiation (1 kr/min) on the conidia of a genetically marked dikaryon of *Neurospora* showed that two different classes of *ad-3* mutations were recovered: (1) *ad-3*^{R} mutations that increase linearly with dose, and (2) *ad-3*^{IR} mutations that increase as the square of the dose. Data from further analyses[2] support the hypothesis that *ad-3*^{R} mutations result from intragenic alterations, whereas *ad-3*^{IR} mutations result from such extragenic alterations as chromosome deletions. All genetic data are consistent with the hypothesis that *ad-3*^{IR} mutations result from the cooperation of two separate events outside the *ad-3A* or *ad-3B* locus which are not detectable separately as *ad-3* mutations. Since it has been shown that the yield of two-hit chromosome aberrations can be modified by changing the dose rate to give sufficient time for recovery from radiation damage, this approach has been used to investigate the effect on mutation induction in the *ad-3* region. In theory, with a decreased dose rate the selective elimination of *ad-3*^{IR} mutations should cause a decrease in the overall frequencies of forward mutation in the *ad-3* region.

Methods. – Conidia of a genetically marked balanced dikaryon (described previously[1]) were x irradiated (250 kvp, 30 ma, 3 mm of Al added filtration) at dose rates of 1 kr/min or 10 r/min to give total exposures of 5, 10, 20, and 40 kr. Total exposures were confirmed with LiF dosimeters. The experiment was performed at 28°C rather than at 0°C[1] to increase the metabolic

activity of the conidia[3] and to enhance the probability for recovery from radiation-induced damage. After treatment, conidia were plated in complete medium to determine the overall survival (of both heterokaryotic and homokaryotic conidia) and in minimal medium to determine the survival of the heterokaryotic fraction. Aliquots were also inoculated into flasks to determine the frequency of mutations in the *ad-3* region by the direct method.[4] In the first series, conidia irradiated with a dose rate of 1 kr/min were plated or inoculated into flasks immediately after irradiation; in the second series, identical samples (irradiated with a dose rate of 1 kr/min) were held at room temperature (24°C) for 66 hr before plating and inoculation into flasks; in the third series, conidia irradiated with a dose rate of 10 r/min were plated or inoculated into flasks as soon as each of the four exposures had been completed. Comparison of the first two series would show the effects of postirradiation storage; comparison of the second two series would show the effect of a 100-fold difference in dose rate.

Results. – *Dose-Rate Effects on Survival.* – The overall survival of both heterokaryotic and homokaryotic conidia in complete medium was unaffected by variation in dose rate or postirradiation storage, but a difference was found in the survival of heterokaryotic conidia in minimal medium as shown in Fig. 3.1.1. No difference in the survival of heterokaryotic conidia was obtained with postirradiation storage, but a striking difference was found with variation of dose rate. The survival curves show that reducing the dose rate from 1 kr/min to 10 r/min gives a dose reduction factor (DRF) of 2.6. In both cases the semilogarithmic plots on the survival curves extrapolate back to 100% survival, showing that inactivation of heterokaryotic conidia requires only a single hit at either dose rate.

Dose-Rate Effects on Forward-Mutation Frequencies. – The forward-mutation frequencies obtained with each of the three treatments are shown in Fig. 3.1.2. Earlier studies with a dose rate of 1 kr/min in an experiment at 0°C[1] showed that the yield y of *ad-3* mutants at any x-ray dose x may be described by the equation $y = ax + bx^2$, where ax equals the yield of *ad-3*^{R} mutants and bx^2 equals the yield of *ad-3*^{IR} mutants. This equation describes an induction curve for *ad-3* mutations which, on a logarithmic plot, has an initial slope of 1.0 at low doses (where the frequency of *ad-3*^{IR} mutations is negligible) and changes to a slope of 2.0 at high doses (where the frequency of *ad-3*^{R} mutations is negligible). Departure from the forward-mutation frequencies expected with 1 kr/min occurred with doses of 20 kr or higher at 24–28°C, indicating progressive preferential inactivation of conidia with mutations in the *ad-3* region.

Dose-Rate Effects on the Spectrum of ad-3 *Mutations.* – The characterization of the *ad-3* mutations in each of the three series is incomplete with regard to reparability (*ad-3*^{R} vs *ad-3*^{IR}) but complete with regard to *ad-3* genotype. Since *ad-3A ad-3B* double mutants are always irreparable,[1] the effect of variation in dose rate on *ad-3*^{IR} mutations can be estimated by comparing the frequencies of *ad-3A ad-3B* double mutants at each exposure. The frequency of double mutants is higher at each of the four exposures with a dose rate of 1 kr/min than with 10 r/min. The

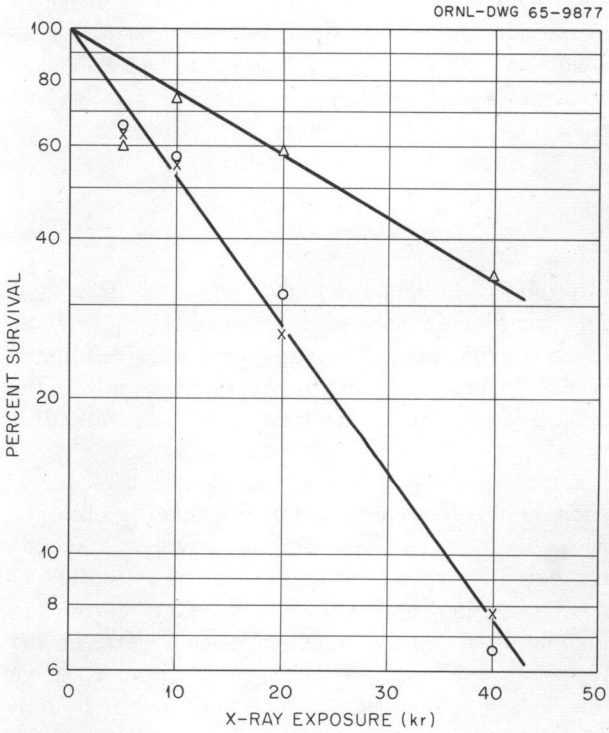

Fig. 3.1.1. Survival of Heterokaryotic Conidia of a Balanced Dikaryon of *Neurospora crassa* in Minimal Medium with Dose Rates of 1 kr/min or 10 r/min. △ = 10 r/min; x = 1 kr/min; O = 1 kr/min + 66 hr of postirradiation storage.

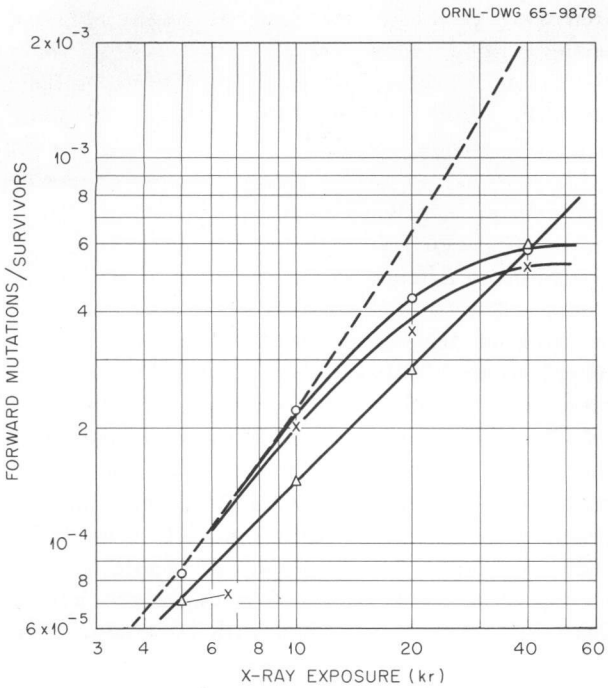

Fig. 3.1.2. Frequencies of Forward Mutations in the *ad-3* Region Obtained with a Balanced Dikaryon of *Neurospora crassa* with Dose Rates of 1 kr/min or 10 r/min. △ = 10 r/min; x = 1 kr/min; O = 1 kr/min + 66 hr of postirradiation storage; dashed line = theoretical curve expected with the formula $y = ax + bx^2$.

differences in these frequencies range from sixfold at the low exposures to threefold at the highest exposures. The variation in the difference is due to the departure from a slope of 2.0 of the induction curves obtained with a dose rate of 1 kr/min.

Effect of Postirradiation Storage. – The difference in the forward-mutation frequencies obtained with the two 1-kr/min series provides evidence for cytoplasmic repair during the 66-hr period of postirradiation storage. This interpretation is favored since there is no difference between the types of *ad-3* mutations in the two series. Although there is no difference in the survival of heterokaryotic conidia with postirradiation storage, the morphology of the colonies was markedly different in the forward-mutation experiments. The variation in both colony size and density obtained with heterokaryotic conidia irradiated with a dose rate of 1 kr/min largely disappears with postirradiation storage, and the uniformity of colony size and morphology obtained with a dose rate of 10 r/min was more nearly approximated. The difference in colony morphology in the two 1-kr series would enhance the manual recovery of purple colonies with the result that the forward-mutation frequencies obtained with postirradiation storage would be higher.

Discussion. – *Dose-Reduction Factors for Repair of Chromosomal Damage.* – Two different lines of evidence indicate that radiation-induced damage is repaired during low-dose-rate irradiation: (1) A higher survival of heterokaryotic conidia with 10 r/min indicates repair of sites of damage which cause nuclear inactivation. (2) A lower induction rate for *ad-3A ad-3B* double mutants with 10 r/min indicates repair of sites of damage which cooperate in pairs to produce this class of *ad-3*^{IR} mutations. The dose-reduction factors for these two phenomena are identical (2.6 for nuclear inactivation and 2.55 for production of *ad-3A ad-3B* double mutants), suggesting that they result from the same type of genetic damage (presumably chromosome breakage) and respond to the same mechanism of repair.

Relationship Between DRF and Mutation Frequency. – If we assume that this damage is chromosome breakage, then the relative frequency of chromosome breakage (F_b) with each dose rate can be estimated from the DRF as follows:

$$\frac{F_b\ (1\ \text{kr/min})}{F_b\ (10\ \text{r/min})} = \text{DRF}\ .$$

Since *ad-3*^{IR} mutations result from the cooperation of two such breaks, the ratio of frequencies of *ad-3*^{IR} mutations (F_m) is equal to the DRF squared:

$$\frac{F_m\ (1\ \text{kr/min})}{F_m\ (10\ \text{r/min})} = \text{DRF}^2\ .$$

With this equation, the DRF value for inactivation of heterokaryotic conidia, and the forward-mutation frequencies obtained with 1 kr/min, the forward-mutation frequencies predicted with a dose rate of 10 r/min are in excellent agreement with those obtained experimentally.

Dose-Rate Effects on Reparable Mutations. – No effect of variation in dose rate on the frequency of *ad-3*^{R} mutations can be detected in present data. The differences in the frequency of forward mutation can be accounted for solely on the basis

of the differences in the frequencies of *ad-3*^{IR} mutations. It is expected that genetic analyses in progress will provide more information on this point, as well as information on any qualitative effects on the spectrum of *ad-3B*^{R} mutations that may be associated with variation in dose rate.

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3.2 COMPARISON OF THE RECESSIVE LETHAL DAMAGE IN IRREPARABLE *ad-3* MUTATIONS

F. J. de Serres

Genetic analysis of the extent and type of functional inactivation in irreparable *ad-3* mutations (*ad-3*^{IR}) has been continued with the use of a tester set discussed in a previous report.[1] The number and classification of these x-ray-induced mutations are as follows: 33 *ad-3A*^{IR}, 64 *ad-3B*^{IR}, 92 (*ad-3A ad-3B*)^{IR}, 10 (*ad-3A ad-3B nic-2*)^{IR}, and 4 (*ad-3B nic-2*)^{IR}. Because x-ray-induced *ad-3*^{IR} mutations increase as the square of the dose and since they will not grow as homokaryons on adenine-supplemented media, the assumption was made that they result from such extragenic alterations as chromosome deletions. This would mean that other loci closely linked would show simultaneous inactivation in mutations originally classified as *ad-3*. This expectation was verified in exploratory homology tests reported previously,[1] where it was possible to demonstrate that the functional inactivation in *ad-3*^{IR} mutations extends beyond the boundaries of the *ad-3A* or *ad-3B* cistron.

Mapping the type and extent of the functional inactivation in the *ad-3*^{IR} mutations, as well as testing for allelic complementation[2] among the *ad-3B*^{R} mutations recovered after x-ray treatment, will serve to characterize the genetic effects of this mutagen. Such analyses will be used to compare the genetic effects of other radiations or chemicals with the dikaryon test system.

Not all the homology tests have been completed on each of the 203 x-ray-induced mutations in this analysis. The results of these tests will be presented diagramatically in a subsequent report. Present data permit a comparison of mutations originally classified as *ad-3A*^{IR} or *ad-3B*^{IR}. As discussed previously,[3] *ad-3* mutations that do not grow as homokaryons on adenine-supplemented media are tentatively classified as *ad-3*^{IR}. Homology tests with strains which carry deletions for the *ad-3A* locus (strain 12-7-215), the *ad-3B* locus (strain 12-5-182), or the *ad-3* and immediately adjacent regions (strain 12-1-18) are then made to determine whether such mutations are actually *ad-3*^{IR} or whether they are *ad-3*^{R} mutations with recessive lethal damage (*RL*) elsewhere in the genome. The nature of the tester strains makes it possible to determine whether this *RL* damage is closely linked or whether it is located elsewhere in the genome.

The sites of *RL* damage in *ad-3*^{R} + *RL* mutations might be expected, a priori, to be distributed at random over the whole genome. The deletion in tester 12-1-18 is estimated to cover about 10% of linkage group I (the smallest of the seven chromosomes in the complement) and should account for no more than one-seventieth of the genome and probably considerably less. More detailed tests on the 33 *ad-3A*^{IR} and the 64 *ad-3B*^{IR} mutations have shown that 4/33 and 2/64 are actually *ad-3*^{R} + *RL* mutations with closely linked *RL* damage. The total number of *ad-3*^{R} + *RL* mutations with closely linked *RL* damage is 8/21 *ad-3A*^{R} + *RL* mutations and 2/35 *ad-3B*^{R} + *RL* mutations.

The sites of the *RL* damage in 3/8 *ad-3A*^{R} + *RL* mutations with closely linked *RL* damage have been mapped. The first was located to the left of the *ad-3A* locus, the second was in the *ad-3A*–*ad-3B* region, and the third was located to the right of the *ad-3B* locus. Tests to map the *RL* damage in the other five *ad-3A*^{R} + *RL* mutations and the two *ad-3B*^{R} + *RL* mutations are in progress.

One possible explanation for a nonrandom distribution of *RL* damage in *ad-3*^{R} + *RL* mutations is that there is not a completely random distribution of ionizations in the cell with acute x irradiation (1000 r/min). Thus, although some *ad-3*^{R} + *RL* mutations result from two separate hits, others may result from a cluster of ionizations from the same electron track so that they are actually

"single-hit" in origin. If the difference in the forward-mutation rates at the *ad-3A* and *ad-3B* loci reflects a difference in locus size, then this nonrandom distribution might be expected to have a more pronounced effect on the production of *ad-3A*^{R} + *RL* mutations with closely linked *RL* damage than the same type of *ad-3B*^{R} + *RL* mutations.

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3.3 DISTRIBUTION OF THE ADENINE-THYMINE AND GUANINE-CYTOSINE PAIRS AT THE MUTANT SITE AMONG NONCOMPLEMENTING MUTANTS AND MUTANTS WITH POLARIZED OR NONPOLARIZED COMPLEMENTATION PATTERNS, IN THE *ad-3B* MUTANTS OF *NEUROSPORA CRASSA*

H. V. Malling F. J. de Serres

Hydroxylamine (HA) has been found to be mutagenic in *Neurospora,*[1] and, from a comparative study of the reversibility of mutants in the tester set known to revert with nitrous acid (NA), it seems likely that HA preferentially induces transitions from guanine-cytosine (GC) to adenine-thymine (AT). This means that base-pair-transition mutations in *Neurospora* can now be characterized as to whether they have an AT pair or a GC pair at their mutant site. The earlier attempt[2] to identify the genetic alteration leading to non-complementing mutants and mutants with polarized or nonpolarized complementation patterns can now be further extended by identifying the two types of base-pair transitions.

Identification of the genetic alterations in individual mutants at the molecular level in *Neurospora* is accomplished by measuring the reversion frequencies after treatment with HA, NA, EMS (ethyl methanesulfonate), or ICR-170 (a monofunctional acridine mustard). The base-pair-substitution mutants are characterized by being revertible with one, two, or three of the first three compounds, the base-pair-insertion and -deletion types of mutants by being revertible with ICR-170 and in some cases also with EMS.

Two more classes have been found: (1) those mutants which do not revert at all (the genetic alterations in those mutants may be due to several changes in the base-pair sequence), and (2) those mutants which under the treatment conditions used for the four mutagens do not give a significantly higher reversion frequency than the control (the genetic alteration in these mutants is due to some, as yet unidentifiable, genetic alterations).

From Table 3.3.1 we can see that mutants with a nonpolarized complementation pattern are exclusively base-pair substitutions and that mutants resulting from a base-pair insertion or deletion

Table 3.3.1. Percentage Distribution of the Different Genetic Alterations Producing Mutants with Polarized or Nonpolarized Complementation Patterns and Noncomplementing Mutants

The numbers in parentheses are the totals. BPS denotes base-pair substitutions; +/– denotes base-pair insertions and deletions.

Complementation Pattern	Type of Genetic Alteration			
	BPS	+/–	Spontaneously Revertible	Nonrevertible
Nonpolarized	100 (61/61)	0	0	0
Polarized	56 (14/25)	0	20 (5/25)	24 (6/25)
Noncomplementing	54 (23/43)	23 (10/43)	7 (3/43)	16 (7/43)

occur only among the noncomplementing mutants. Mutants which are only spontaneously revertible or which do not revert occur with the highest frequency among mutants with polarized complementation patterns.

Table 3.3.2 gives the distribution of base-pair-substitution mutants which revert after HA treatment, and therefore are assumed to have a GC pair at the mutant site, and those which do not revert after HA treatment and therefore are assumed to have an AT pair at the mutant site.

The mutants with a nonpolarized complementation pattern have predominantly GC pairs at their mutant site, whereas base-pair-substitution mutants with a polarized complementation pattern and noncomplementing mutants have a distribution of approximately 50% AT pairs at their mutant site. Very recently, the base composition of the codons for the triplets which terminate the formation of the polypeptide chain (nonsense triplets) has been identified in *E. coli.*[3,4] The codons are UAG and UAA. The DNA which is equivalent to these codons must contain a high proportion of AT pairs.

If we now assume that the base composition for the terminating codons of the polypeptide chain is the same in *Neurospora* and *E. coli*, then the high frequency of AT pairs at the mutant site among the mutants with polarized complementation patterns and noncomplementing mutants could easily be explained by assuming that mutants which only form fragments of the polypeptide chain never have a nonpolarized complementation pattern. We can, therefore, conclude: (1) that nonsense mutations give rise to mutants with polarized complementation patterns and noncomplementing mutants, (2) that base-pair-insertion or -deletion mutations only occur among the noncomplementing mutants, and (3) that mutants with unidentified genetic alterations occur only among mutants with polarized complementation pattern and noncomplementing mutants.

Table 3.3.2. Percent Distribution of the AT Pairs and GC Pairs Among the Base-Pair-Substitution Mutants That Have Either a Nonpolarized or a Polarized Complementation Pattern or Are Noncomplementing

The numbers in the parentheses are the totals.

Complementation Pattern	Type of Genetic Alteration on the Mutant Site	
	AT	GC
Nonpolarized	7 (4/57)	93 (53/57)
Polarized	43 (6/14)	57 (8/14)
Noncomplementing	52 (11/21)	48 (10/21)

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3.4 PHOTOREACTIVATION IN *NEUROSPORA CRASSA*

B. J. Kilbey

Experiments have been conducted in order to establish the existence of and the conditions for the maximum expression of photoreactivation of survival in *Neurospora crassa*. The strain used is the wild-type stock 74-OR23-1A previously used to obtain ultraviolet-induced adenine mutants.[1] The findings were as follows:

1. Photoreactivation is readily demonstrated in this strain. Conidia inactivated with graded doses of ultraviolet (UV) light at a dose rate of 18.5 ergs sec^{-1} mm^{-2} were photoreactivated in a water suspension or in plating medium on petri dishes. The light sources included daylight and fluorescent lamps, but in most experiments the conidia were photoreactivated in aqueous suspension at 35°C in a water bath. The tubes containing the suspensions were supported around a 300-w incandescent lamp suspended just above the bath. The distance between the tubes and the lamp was constant and about 6 cm. During the exposures to both UV and visible light the suspensions were stirred. In all cases the photoreactivable sector was reasonably constant over the whole range of UV doses at 0.35.

2. Conidia irradiated and exposed to visible light in this way required a minimum time for maximum photoreactivation, which depended to some extent on the degree of initial inactivation by UV. At 45% survival a maximum amount of photoreactivation was attained in 15 min, but at 2% survival 30 min were required for maximum photoreactivation. The visible light itself was never shown to inactivate the conidia during periods of illumination of up to 90 min.

3. The potentially lethal damage was fixed in the conidia at a rate that depended on the temperature at which the conidia were incubated following UV irradiation. Conidia incubated in the dark on plating medium at 30°C for various periods before illumination with visible light started to lose their photoreactivability almost immediately. The process was complete after about 120 min. Conidia from the same experiment that were incubated in the dark at 4°C, on the other hand, retained their photoreactivability for at least 24 hr. They had lost it, however, by 84 hr.

4. During the course of these experiments it became clear that there is no immediately obvious dark repair process. The colony counts were the same whether conidia were held in suspension in the dark for up to 45 min at 35°C or plated as quickly as possible. However, if such a process existed, which was very rapid, it would not have been detected in these experiments. It is of interest to note that no evidence for photoprotection was obtained, and a tenfold alteration in the UV dose rate was without effect on the dose-effect curves for survival. Dose fractionation experiments have still to be carried out.

It appears very likely that the photoreactivation demonstrated here and by other workers with *Neurospora*[2] is the result of an enzymatic process similar to that demonstrated for *Escherichia coli* by Rupert *et al.*[3] However, experiments are in progress to try to establish this with certainty. *Neurospora* may be very useful for a study of photoreactivation effects since there are apparently no dark repair processes to interfere with their analysis. These data have been used to design and carry out experiments to study the quantitative and qualitative effects of photoreactivation on UV-induced mutation to adenine auxotrophy in *Neurospora*. The results of the mutation experiments are incomplete and will be reported at a later date.

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3.5 THE ACID-SOLUBLE NUCLEOSIDES AND NUCLEOTIDES FOUND DURING THE GERMINATION OF *NEUROSPORA* CONIDIA

H. V. Malling I. L. Cameron[1]
W. D. Fisher[2]

The different stages in the cycle of nuclear division have very different sensitivities to mutagens. These sensitivities have been correlated with the synthesis of DNA. The synthesis of DNA, in turn, is dependent on the pool of the free nucleotides in the cell. For a better understanding of the mutation induction and expression in *Neurospora* conidia, we feel, therefore, that it is important to study the change in the pool of free nucleosides and nucleotides in the cell during the germination and growth.

Freshly harvested conidia of wild type 74A were incubated for various times in Fries minimal medium supplemented with glucose. The percentages of germination were 7, 22, and 58 after 3, 4, and 5 hr of growth respectively. A 20% increase in packed cell volume was first noted after 5 hr; by 10 hr the packed cell volume had increased sevenfold.

The chromatographic separation methods for nucleosides and nucleotides as described by Anderson *et al.*[3] have been applied to cold-acid-soluble extracts of freshly harvested conidia and to extracts of conidia incubated for 4 and 10 hr in growth medium. Spectrophotometric analysis of the column effluent at 260 and 280 mμ shows 25 peaks. Comparison of the peak patterns, as well as the 260/280 mμ absorbance ratios, with those of mixtures of known purines and pyrimidine bases, nucleosides, and nucleotides permits presumptive identification of the nucleotide derivatives from *Neurospora*.

The content of nucleosides and nucleotides in nongerminated conidia is comparatively low. However, during the first 4 hr of incubation in growth

medium the content of all the individual nucleosides and nucleotides increases. When the hyphae are well developed, there is a considerable decrease in all the nucleosides and nucleotides when expressed per unit volume of packed cells.

The most dramatic change was found among the uridine derivatives, which show a substantial increase at 4 hr after inoculation, a period immediately prior to hyphal growth. Thereafter, when the hyphae are well developed, the uridine derivatives decrease to a very low level. UDP-*N*-acetyl glucosamine and UDP-glucose are known to be precursors for the cell walls in yeast, and therefore the drop in the uridine derivatives implicates the possible utilization of these compounds as precursors in the formation of new cell walls in *Neurospora*.

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Drosophila Cytology and Genetics

D. L. Lindsley

Radiation Genetics (4.1–4.3)

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Chromosomal Behavior and Biochemical Genetics (4.4)

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Pseudoallelism and Immunogenetics (4.5)

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4.1 REVISION OF *THE MUTANTS OF DROSOPHILA MELANOGASTER*

D. L. Lindsley
E. H. Grell
I. Lucille Norton
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 2½ years ago, and we have been writing for approximately 20 months. To date, detailed descriptions have been prepared of all mutations that have been described; this represents well over half the material that will go into the book. Descriptions of the chromosome aberrations have been completed. When the editing of the chromosome aberrations is 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 to prepare tables ordered on any characteristic or combination thereof. The machine program has been completed and seems to be in good working order. As we have anticipated in previous semiannual reports, we anticipate that in the next semiannual report we will be able to state that our part of the task is finished.

4.2 CROSSOVER-SUPPRESSING TRANSLOCATIONS IN *DROSOPHILA MELANOGASTER*

P. A. Roberts

A technique whereby all major chromosome arms can be simultaneously screened for crossover-suppressing aberrations involves the use of a stock

with markers spaced approximately 50 crossover units apart. Males were exposed to 4000 r of x rays and mated to marker females, and individual females were backcrossed to marker males. Stocks were made of the crossover suppressors, and the polytene chromosomes of 75 of these rearrangements have been examined. In addition to the expected para- and pericentric inversions, a number of crossover-suppressing translocations, both reciprocal and insertional, have been recovered. When an element of a translocation has a proximal break, recombination within the arm is little affected, but when the break is in the distal two-thirds of an arm, recombination within this arm can be reduced to very low values, comparable to that of an inversion heterozygote. The establishment of homologous associations *distally* is apparently a prerequisite for regular meiotic exchanges within a chromosome arm.

4.3 TIMING SPERMATOGENESIS IN *DROSOPHILA MELANOGASTER* WITH SCINTILLATION COUNTING OF TRITIATED THYMIDINE

W. E. Trout III

In studies of the effect of mutagens on stages of spermatogenesis in Drosophila it is desirable to know how the time of appearance of mature sperm, which were in different stages at the time of treatment, has been affected. Studies by Chandley and Bateman,[1] using tritiated thymidine and autoradiography, have indicated that labeled mature sperm do not appear until ten days after injection. The present preliminary studies, using scintillation counting of the inseminated females, have suggested that a label different from unincorporated thymidine is transferred about the eighth day after injection, indicating either that the spermatogenetic cycle is shorter under the conditions used or that some labeled component of the ejaculate other than the sperm head is transferred and was not detected by the autoradiographic method. At present, efforts are being made to improve the scintillation counting method by varying the mating scheme of the males, the age of the males at injection, and the method of preparation of the inseminated females for counting.

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4.4 CHROMOSOME SIZE AND RADIATION-INDUCED NONDISJUNCTION

Rhoda F. Grell W. F. Kirschbaum[1]
E. R. Muñoz[1]

It is well established that ionizing radiation leads to an increased frequency of abnormal distribution of chromosomes. When the irregular segregation occurs at meiosis, the gametes that result either carry an extra chromosome or lack a chromosome. Gametes of this kind, with unbalanced chromosome sets, are said to be aneuploid; the process which results in such aneuploid types is termed nondisjunction, and the consequence of aneuploidy is, in general, lethality before maturity is reached.

Although both centromere malfunction[2] and chromosome stickiness[3] have been tentatively suggested as explanations for the failure of irradiated homologs to separate during a meiotic division, the mechanism responsible is, in fact, unknown. One question that may now be answered is whether chromosome size affects the frequency of radiation-induced nondisjunction. Heretofore, such nondisjunction studies in the Drosophila female have been largely confined to the X chromosomes, where both nondisjunctional products, that is, the XX and 0 types, are fully viable and are recoverable in half the zygotes. By contrast, nondisjunction of any pair of autosomes results in reduced or complete inviability of at least one of the two products. A technique is now available which permits two small chromosomes to be added to the genome with no ensuing inviability. The small chromosomes consist of an extra, free fourth chromosome and a free X duplication, equivalent in length to chromosome 4. The extra chromosomes behave, for segregation purposes, as if they were homologs and, at the first meiotic division, move to opposite poles ~99.9% of the time. Thus it becomes possible in the same female to compare the effect of radiation on the nondisjunction of a pair of X chromosomes and on a pair of chromosomes approximately one-tenth the length of the X's. In the present experiments, newly eclosed females carrying the two extra chromosomes were exposed to 4000 r of x rays, held for 2 days before mating, and brooded daily thereafter for 12 days.

Results and Discussion. – Table 4.4.1 gives the nondisjunction frequencies for the X chromosomes (column 3) and for the two small chromosomes

Table 4.4.1. Effect of 4000 r of X Rays on Nondisjunction of the X Chromosomes and of Two Regularly Segregating Nonhomologs, One-tenth the Length of the X's

Brood Day	Number	X Nondisjunction (%)	Dp, 4 Nondisjunction (%)
1	1,380	1.4	1.9
2	2,221	1.9	2.5
3	2,979	1.1	2.5
4	4,999	0.9	1.9
5	6,106	1.3	1.8
6	8,021	1.4	1.8
7	6,772	1.5	1.6
8	6,717	0.5	0.6
9	6,535	1.6	0.8
10	6,871	0.9	0.4
11	6,296	0	0.8
12	3,410	1.3	0.3
	62,307[a]	1.1[b]	1.3[b]

[a]Total.
[b]Average frequency.

(column 4). The values shown have been corrected for the control rates. Day 1 represents oocytes irradiated at stage 7, and succeeding days represent irradiation of progressively earlier oocyte stages and of oogonia.

The average nondisjunction frequency for the 12 broods is 1.1% for the X chromosomes and 1.3% for the X duplication and the fourth chromosome. In both cases a sharp decrease is observed in the eighth-day brood. From independent evidence,[4] this brood probably represents premeiotic interphase oocytes at the time of treatment. This may mean that the mechanism responsible for nondisjunction is related to that responsible for structural chromosome changes, since the frequency of the latter is also greatly reduced if irradiation occurs during interphase.

The present data show no correlation between nondisjunction frequency and chromosome size. These observations fail to support the supposition that chromosome stickiness is responsible for the failure of chromosome separation if it is assumed that the opportunity for adhesion is related to chromosome length. The results are more compatible with the hypothesis that centromere misbehavior is responsible for nondisjunction.

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4.5 DATA SUPPORTING NOTCH AS A CISTRON

W. J. Welshons J. W. Day

At the Notch locus, recessive lethal and recessive visible mutants have been used in the fine-structure analysis, leading to the conclusion that the Notch locus in Drosophila is best described as a cistron. The recessive lethals (*N* mutants) are believed to be amorphs lacking virtually all genetic activity. Presumably, a mutant change at the DNA level prevents either the formation of a messenger RNA (mRNA) or the translation of an mRNA into a functional polypeptide. A recessive visible like split (*spl*) appears to be a mutant that has retained some genetic activity; hence the mutant DNA acting through the intermediary RNA's causes the formation of a protein or polypeptide that is at least partially functional. If the interpretation of the mutants is correct and if one is dealing with a gene or cistron functioning in the formation of a single polypeptide, then, in the case where the recessive lethal *N* is coupled with the recessive visible *spl*, it should be impossible to detect a partially functional "*spl*-protein" since its presence is already precluded by the existence of the amorphic recessive lethal mutant (*N*) on the same chromosome and within the same gene.

A coupled double-mutant combination was obtained by inducing the recessive lethal N^{64d6} mutant on a chromosome that already carried the recessive visible pseudoallele *spl*. Heterozygous females $z\ N^{64d6}\ spl/+++$ were mated to $+;\ E\text{-}spl/+$ males heterozygous for the dominant enhancer of the recessive visible *spl* mutant. A large number

of Notch females from the cross were examined for the expression of *spl* since it had been shown previously that even females with one dose of *spl* and two doses of the wild allele strongly express the split phenotype when the enhancer is present. Although half the Notch females should carry *E-spl*, none of the animals showed even a slight indication of a split phenotype.

To be certain that the chromosome carried the coupled mutants N^{64d6} and *spl*, *z* ++ N^{64d6} *spl* +/+ w^a fa^g ++ *rb* ♀♀ were mated to w^a *fa* fa^{no} *rb* ♂♂ in order to separate the pseudoalleles facet-glossy (fa^g), Notch^{64d6} (N^{64d6}), and split (*spl*). Recovery of recombinant chromosomes *z* ++++ *rb* indicated that N^{64d6} was localized to the right of fa^g, and one + w^a fa^g + *spl* + proved that *spl* was present and placed the mutant site of N^{64d6} between fa^g and *spl*.

The failure to produce a split phenotype in the presence of *E-spl* when *spl* is coupled to an *N* mutant supports the interpretation of Notch as a cistron. Similar observations might be made if Notch were a polycistronic operon; however, data of another nature fail to support this alternative.

Cytochemistry and Cell Reproduction

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Yeast Genetics (5.1, 5.2) R. C. von Borstel[a] T. R. Manney K. C. Atwood[b] Margaret D. Schellinger Myra Berman[c] Sue Ellen Frederick[c] Gladys S. Van Pelt[d]	**Cell Biochemistry (5.3–5.8)** I. L. Cameron Betty M. Wysinger
Insect Cytology and Genetics J. G. Carlson[b] Mary Esther Gaulden[b] Anna R. Whiting[a,b] P. W. Whiting[b] Diane J. Goins[a]	*Participation in Cooperative Projects:* AEC-NASA Space Biology Program (11.4)
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5.1 SPONTANEOUS REVERSION RATE MEASUREMENTS OF AUXOTROPHIC MUTANTS OF *SACCHAROMYCES CEREVISIAE* DURING VEGETATIVE GROWTH

R. C. von Borstel Martha J. Bond[1]
Diane J. Goins

Introduction. – During the course of studying the reversion rates of different auxotrophic mutants of the yeast *Saccharomyces cerevisiae*, it was found that certain mutant strains undergo starvation death on limiting media. The phenomenon of starvation death has been observed previously[2] and has been attributed to a single gene that causes a yeast cell to be sensitive to starvation on amino acids but not on adenine or glucose. It appears that when cells of a sensitive strain die they add amino acids to a limiting culture so that the living cells will continue to grow and divide. When revertant colonies are sought by placing cells on a limiting medium, the constant low level of growth and budding of cells growing on the remains of dead cells continually adds new revertant cells to cultures. Therefore it is extremely difficult to determine the reversion rates of mutants in the sensitive strains.

Results. – *Reversion Tests in Growth-Limiting Media.* – These tests were performed by placing 1000–10,000 mutant cells/ml in a medium which would limit growth at approximately 10^7 cells/ml, with the auxotrophic requirement of the mutant as the growth-limiting factor. When reversions occur, growth normally proceeds to a cell concentration of 2×10^8 cells/ml. The inoculum was distributed in 1-ml aliquots in each of approximately

1000 compartments in the multicompartmented plastic boxes of de Serres. Reversions were scored by the excessive growth observed in some of the compartments. These mutant compartments usually begin to appear after the fourth day at 30°C, and a plateau is reached by the tenth day (lower curve, Fig. 5.1.1).

The mutation rate is determined from the cell number in the nonmutant compartments and the proportion of compartments without mutants (when the plateau is reached) by the method of Luria and Delbrück.[3] The reliability and accuracy of this method can be seen from the data in Table 5.1.1. When the same adenine-requiring mutant (ad_{6-45}, kindly supplied by E. W. Jones) was used under slightly different conditions of supplementation of amino acids and bases to the media, essentially identical mutation rates were obtained.

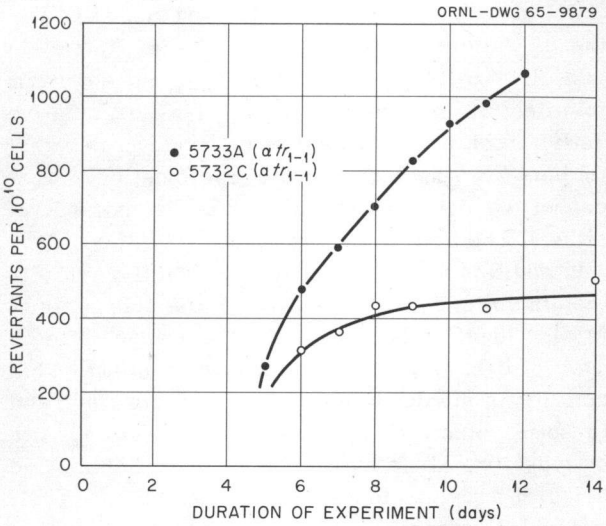

Fig. 5.1.1. Time of Appearance of Revertant Colonies from *Tryptophan*$_{1-1}$ Haploids (30°C).

Reversion Tests by Transfer from Nonlimiting to Growth-Restricting Media. – Unfortunately, an occasional mutant strain does not reach a plateau during reversion-compartment production (top curve, Fig. 5.1.1). We presume that the cells dying from starvation contribute amino acids to the medium, causing the slow turnover of cells and continued reversion production. In order to obtain mutation rates for such strains, we grew 15 tubes of cells overnight (to approximately 10^8 cells/ml) on an enriched yeast-extract medium containing all the essential amino acids and bases (YEPD medium), and transferred these cells at a concentration near 10^7 cells/ml to 15 different multicompartmented boxes containing none of the auxotrophic requirements of the mutant. Compartments containing revertants could be seen in one day and were counted on the third day, before starvation-killed cells could contribute essential requirements for cell growth and the associated reversion production. After the third day the newly arising revertants begin to contribute to the number of revertant compartments.

The reversion rate is determined for each box, and the value taken is selected by the method of the median.[4] For the ad_{6-45} mutant strain tested

Table 5.1.1. Spontaneous Reversion Rates of the ad_{6-45} Mutant of J2 Strain of *S. cerevisiae*

Conditions	Number of Compartments	Cells per Compartment	Number of Nonmutant Compartments	Reversions per Nucleus per Generation
		$\times 10^6$		$\times 10^{-9}$
Auxotrophic requirements only; 1 γ/ml adenine	998	3.91	984	3.61
Auxotrophic requirements only; 2 γ/ml adenine	1000	8.43	969	3.72
10 amino acids and bases added; 2 γ/ml adenine	1000	9.20	967	3.64

in this way in one experiment, the mutation rate was found to be 4.8×10^{-9} reversion per nucleus per generation.

Discussion. – The spontaneous reversion rate as determined by transfer from nonlimiting to growth-restricting media has a larger variance than the reversion rate determined by tests in growth-limiting media. This is simply because the sample size can be 15 times larger in the latter case than in the former for the same amount of work. Nevertheless, these methods provide accurate and reliable estimates of the spontaneous reversion rates for auxotrophic mutants of any strain of yeast.

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5.2 REGULATION OF THE STABILITY OF AN ENZYME

T. R. Manney Margaret D. Schellinger

Introduction. – In the course of a search for conditions that would repress or derepress the level of the enzyme tryptophan synthetase (TSase) in yeast, we found an effect that could provide a biochemical basis for the regulation of the rate at which this enzyme (and possibly others) is inactivated in the cell. We have found that the relative specific activity (units per mg of protein) of this enzyme is only slightly influenced by the composition of the culture medium, whereas its stability is profoundly affected. The stability of this enzyme is under the control of a bipartite system that consists of one or more enzymes that can degrade it and at least two agents that inhibit this degradation. Control appears to be achieved primarily through modulation of the concentration of these inhibitors.

Experiments and Results. – When a haploid strain of *Saccharomyces cerevisiae* (S288C) was grown in minimal medium, the TSase activity extracted from it decayed exponentially with a half-life of less than 1 hr at 37°C. When the same strain was grown in a complex medium (yeast extract, peptone, and dextrose), however, its TSase activity was quite stable with a half-life greater than 10 hr at 37°C. When cells grown in minimal medium were washed and grown for short periods of time in complex medium, the shape of the inactivation curve remained exponential, but the half-life became greater ($T_{1/2}$ = 85 min after 110 min of incubation in complex medium). If two kinds of TSase, unstable and stable, were made in the two kinds of medium, the curves should have been biphasic rather than the simple exponential. These results suggested that changes of other constituents in the cell are responsible for the differences in the stability of this enzyme.

We have been able to isolate an activity from these cultures that will inactivate TSase in partially purified preparations but does not seem to affect it in crude extracts. This degradative activity can be isolated from cells grown in either medium by ammonium sulfate fractionation, but it cannot be detected directly in crude extracts of either. The activity is destroyed by boiling.

In addition we have isolated a protective activity that inhibits this degradation. While this activity is also found in both cultures, there appears to be more of it in cells grown in complex medium. This activity is stable to boiling and has been purified to some extent. It appears to be associated with at least two kinds of molecules, one that is acid soluble and one that is acid insoluble. Their molecular weights appear to range from greater than 5000 to less than 50,000, judging from gel filtration on several different grades of Sephadex.

It would appear that the changes in the levels of the protective factors can account for the observed differences in the stability of TSase. The isolation and quantitation of the degradative activity are not precise enough, however, to rule out some changes in its concentration in the two kinds of culture. In any event, the system provides the cell with a means for rapidly altering the stability of at least this enzyme (TSase) in response to changes in the growth medium.

5.3 A PERIODICITY OF ^{3}H-THYMIDINE INCORPORATION INTO CYTOPLASMIC DNA DURING THE CELL CYCLE OF *TETRAHYMENA PYRIFORMIS*

I. L. Cameron Betty M. Wysinger

Reports indicate that ^{3}H-thymidine is incorporated into a DNase-sensitive material in the cytoplasm

of the ciliate *Tetrahymena pyriformis*. It is now known that most of this cytoplasmic DNA is localized inside the mitochondria of this species.[1] Furthermore, this cytoplasmic DNA appears to be stable and conserved during cell growth and reproduction.[1]

It is the purpose of this communication to establish if there is a relationship between cytoplasmic DNA replication and the DNA replication in the cell's micro- or macronucleus. *Tetrahymena pyriformis* strain HSM is known to replicate its micronuclear DNA during a half-hour period at the time of cell division, whereas the macronucleus replicates DNA for about an hour during the middle of the cell cycle. Thus the two nuclei replicate DNA independent of each other.

The cells were grown axenically in a defined medium plus 0.04% proteose peptone. The cells have a generation time of 225 min in this medium. Cultures of these cells, in log growth, were exposed to ^{3}H-thymidine, 20 μc/ml (specific activity of 6.7 curies per millimole) for 15 or 30 min, air dried on slides, washed with cold 0.5 *N* trichloroacetic acid and 3:1 ethanol–acetic acid, and prepared for autoradiography. At least two weeks of autoradiographic exposure are necessary to increase cytoplasmic grain counts to a measurable level.

Both cytoplasmic and nuclear radioactivity were removed by DNase digestion. Analysis was performed on cells which were classified into three groups: (1) with a labeled macronucleus, (2) with a labeled micronucleus, and (3) without nuclear labeling. An ocular grid was placed over an area of cytoplasm, at some distance from either nucleus, and the silver grains counted.

The average grain counts per unit area for seven individual experiments, corrected for background, are listed in Table 5.3.1. The individual and pooled tests of equality of the following categories of cells are shown in Table 5.3.2: (1) without macronucleus labeled vs micronucleus labeled, (2) macronucleus labeled vs micronucleus labeled, and (3) without macronucleus labeled vs macronucleus labeled. When the individual *t* values are pooled as shown in Table 5.3.2, the following conclusions are warranted: The incorporation of ^{3}H-thymidine into cytoplasmic DNA is higher during micronuclear DNA replication than during any other part of the cell cycle. Comparison of ^{3}H-thymidine incorporation into the cytoplasmic DNA of cells without macro- or micronuclear label and into cells with a labeled macronucleus shows no significant difference. With but few exceptions the individual tests of equality in the different experiments showed that the cytoplasmic DNA label is significantly above background throughout the cell cycle.

Short exposure of cells to ^{3}H-thymidine was employed because longer exposures would allow appreciable numbers of cells to pass from one part of the cell cycle to another and would therefore confuse the classification of cells and subsequent interpretations. An increased ^{3}H-thymidine incorporation into cytoplasmic DNA at the beginning of macronuclear DNA replication has been observed in this laboratory and elsewhere.[1] Apparently, this increased incorporation rate is of

Table 5.3.1. Average Grain Count per Unit Area of Cytoplasm

Experiment Number	Labeled Macronucleus	Labeled Micronucleus	Without Nuclear Label
1	4.14	9.12	4.17
2	1.32	2.23	−0.59
3	7.30	9.70	4.40
4	1.70	4.40	2.40
5	13.80	15.80	7.20
6	−0.20	5.20	−0.20
7	9.11	11.50	8.50

Table 5.3.2. Individual and Pooled Tests of Equality (t Values)[a]

Experiment Number	No Nuclear Label vs Micronucleus Labeled	Micronucleus Labeled vs Macronucleus Labeled	No Nuclear Label vs Macronucleus Labeled
1	3.29[b]	3.82[b]	0.017
2	2.88[b]	1.22	2.24[c]
3	2.24[c]	1.07	1.22
4	0.79	1.07	0.28
5	1.58	0.37	1.21
6	3.21[b]	3.21[b]	0
7	2.07[c]	1.45	0.51
Chi square (χ^2) with 14 degrees of freedom	(50.2)[b]	(37.4)[b]	(14.02)
Pooled experiments	0.0005	0.001	$\cong$0.40

[a] $M_i = M_j$ or $\bar{x}_i = \bar{x}_j$; $t = (\bar{x}_i - \bar{x}_j)/\text{S.E.}\ (\bar{x}_i - \bar{x}_j)$.
[b] Significant at 1%.
[c] Significant at 5%.

such short duration that it has been missed by my method of analysis, which sums the grain counts during the entire macronuclear DNA-synthetic period.

Cytoplasmic DNA synthesis takes place during most of the cell cycle. The increased cytoplasmic incorporation of ^{3}H-thymidine which takes place during micronuclear DNA synthesis and which is also observed at the beginning of the macronuclear DNA-synthetic period may reflect an increased rate of cytoplasmic DNA synthesis at these times in the cell cycle. Alternatively, the increased incorporation may reflect a change in the size of deoxyribonucleotide pools which occurs at these two times in the cell cycle. The findings suggest that there is an interdependence between nuclear DNA synthesis and cytoplasmic DNA synthesis.

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5.4 PHYSIOLOGICAL AND STRUCTURAL CHANGES IN THE CELL CYCLE OF REPETITIVELY SYNCHRONIZED *TETRAHYMENA*

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L. H. Elrod[3]

Tetrahymena pyriformis HSM was synchronized by the "one-cold-exposure-per-cycle" method described by Padilla and Cameron.[4] The method involves a 12-hr temperature cycle (2.5 hr at 27°C and 9.5 hr at 12°C). Ninety percent of the cells regularly divide during the last hour of the warm period.

During the 9-hr cold period, certain cellular activities continue, while others are slowed or arrested altogether (see Table 5.4.1). The cellular content of carbohydrate and of DNA increases about 100%; RNA, protein, and Q_{O_2} increase 33, 22, and 11% respectively. Stomatogenesis is completely stopped. In the first 1.5 hr of the new warm period, almost every cell undergoes stomatogenesis. Ribonucleic acid, protein, Q_{O_2}, and DNA

Table 5.4.1. Cellular Content of Protein, RNA, DNA, Carbohydrate, Cellular Respiration Rate, and Stomatogenesis During the Cell Cycle of Synchronized *Tetrahymena pyriformis* HSM[a]

	Amount at the End of the 9-hr Cold (12°C) Period	Percent Increase	Amount After First 1.5 hr of New Warm Period (27°C)	Percent Increase	Amount at Last Hour of Warm Period During Which Cell Number Increases	Percent Decrease
Protein, mg/10^6 cells	0.670	+22	0.875	+31	0.550	−37
RNA, μg/10^6 cells	400	+33	600	+50	300	−50
DNA, μg/10^6 cells	34	+110	35.5	+4	16	−55
Carbohydrate,[b] mg/10^6 cells	0.193	+93	0.179	−7	0.100	−44
Q_{O_2}, μl (10^6 cells)$^{-1}$ hr^{-1}	168	+11	209	+24	155	−26
Number of mouths per cell	1	0	~2	+100	1	−50

[a]Synchronized by the "one-cold-exposure-per-cycle" method (a 12-hr cycle, 9.5 hr at 12°C and 2.5 hr at 27°C). Ninety percent of the cells regularly divide during the last hour of the warm period.

[b]Data from A. A. Barber, W. W. Harris, and G. M. Padilla (submitted to *J. Cell Biol.*, 1965).

increase 50, 31, 24, and 4% respectively. Carbohydrates decrease 7%. During the 1-hr period when the cell number is increasing, RNA, DNA, carbohydrate, and number of mouths per cell drop almost half. This is expected because of the partition of material to the daughter cells. On the other hand, protein and Q_{O_2} do not halve, which suggests that these parameters are increasing during cell division.

It is probably futile to look at any one of the quantities of the above parameters as a "trigger" which initiates cell division. Yet, it seems especially important to understand how an increase in one parameter will either permit, initiate, or release another cellular parameter for the development of an orderly continuation of cell growth and reproduction.

Our results may be interpreted as follows: An increase in DNA templates in one period will allow increased synthesis of RNA in the next period. Increased RNA (perhaps polysomes) will, in turn, allow more protein synthesis in the next period. Accumulation of carbohydrates (or energy reserves) in one period will free the cells' labors for increased respiration, stomatogenesis, and a generally increased synthetic activity in the next period.

Although the different cellular parameters change with different patterns in the cold and warm periods, a balance in chemical and structural elements of the cell appears to be maintained from one cell cycle to the next.

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5.5 AGE-DEPENDENT CHANGES IN THE MORPHOLOGY OF BROWN FAT TISSUE IN MICE

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

Brown fat has recently been recognized as a thermogenic tissue capable of producing a considerable amount of heat in cold-exposed nonhibernating mammals such as the rat, guinea pig, rabbit, and man, and also in these hibernating

mammals during arousal: bat, marmot, and ground squirrel. In rats, brown fat undergoes trophic and physiological responses, indicating its importance to nonshivering cold acclimation. Recent studies have suggested that brown fat may play a very important role in the nonshivering thermogenesis in the newborn of the rabbit, guinea pig, and man.

It has been shown that the newborn guinea pig loses the thermogenic capacity of its brown fat tissue with increasing age. It has also been demonstrated that the newborn mouse quickly loses its ability to tolerate cold exposure with increased age. Finally, workers have shown that the ability of the rat to make a satisfactory nonshivering cold acclimation decreases after the animal is 3 months old. Rats older than 6 to 9 months seldom survive the same cold exposure (personal observation). Because of the importance of brown fat in thermogenesis of mammals and because young nonhibernating mammals soon lose their tolerance to cold and later their adaptability to cold, we undertook this study to see if there were morphological changes in brown fat tissue which could be correlated with the functional changes taking place during the aging of the mammal.

Our observations, which were limited to mice, suggest that the loss of nonshivering thermogenesis in newborns and the inability of adults to make a nonshivering acclimation are correlated with the general age-dependent decrease of the multilocular type of fat cell (several lipid droplets per cell) and an increase in the unilocular type of fat cell (one lipid droplet per cell) in all brown fat tissue.

Methods. – Fourteen male mice of the Swiss albino strain were used. They were born, raised, and killed in the same room under constant temperature (26°C) and lighting (13 hr of light, 11 hr of dark). Water and food (Purina mouse breeder chow) were ad libitum. Animals were killed at various ages from birth to 19 months of age. The average life span of these mice is about 2 years. The mice were killed at 3:00 pm by a blow on the head, and tissues were removed and fixed for 3 days in Bouin-Hollande fluid.

The tissues were embedded in nitrocellulose paraffin, sectioned at 5 μ thickness, and stained with Masson's trichrome.

Observations. – Histological examinations of sections of newborn mice show clearly the anatomical distribution of multilocular brown fat. It is most abundant in the neck region, but one can discern brown fat anlagen in the following specific areas: (1) interscapular, (2) dorsal-cervical, (3) subscapular-axillary, (4) inferior cervical (near the first two cervical vertebrae), (5) superior mediastinal (around the thymus and posteriorly to the roots of the great vessels), (6) suprasternal (abundant in the area of the salivary gland), (7) packed between the thoracic vertebrae and around the thoracic vessels, (8) retroperitoneal between the kidneys, (9) perianal, (10) inguinal, and (11) retrolumbar. In the last three areas the multilocular cells transform almost entirely into unilocular cells during the first month of life. This same transformation takes place in all areas of brown fat, but at different rates in different areas. Within the anlagen, the peripherally located cells and the cells in the areas along the trabeculae are the first to make the multi- to unilocular transformation. The cell diameter more than doubles between 3 and 19 months. Our histological sections of brown fat tissue show an age-dependent transformation from an almost exclusive multilocular cell composition to an almost exclusive unilocular cell composition. Once the cells become unilocular, they continue to grow by increasing their volume.

Discussion. – Multilocular brown fat cells are known to be rich in mitochondria, and the tissue has a high rate of oxidative metabolism. The transformation of multilocular cells into unilocular cells and the continued age-dependent growth of the unilocular cells by lipid deposition ultimately lead to a decrease in the mitochondrial population per unit volume of tissues.

In previous studies, brown fat has been shown to be strongly thermogenic in homoiothermic mammals and is capable of undergoing a trophic response in cold-exposed animals. During cold exposure new multilocular brown fat cells arise by cytogenesis from reticuloendothelial progenitor cells. In addition, the brown fat tissues increase in mass, and the tissue changes from one having unilocular and multilocular cell types to one having almost exclusively the latter. These changes are completed by the time the new thermogenic steady state has developed. This new steady state is not greatly dependent on shivering for heat production.

The rapid loss of tolerance to cold by newborn mammals may be due to the transformation of brown fat from the multilocular to the unilocular

cell type with an associated decrease in the tissue's oxidative capacity, since the times of onset of the two phenomena are closely correlated. Such a correlation may also explain why young adult animals are able to make a nonshivering cold acclimation whereas older animals are not.

Summary. – The multilocular cells of brown fat tissue show an age-dependent transformation to the unilocular cell type. This transformation starts immediately after birth and continues throughout the life of the mouse. This morphological change is correlated with an age-dependent decrease in tolerance and adaptability to a cold environment.

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5.6 SEPARATION OF ACID-SOLUBLE NUCLEOTIDES AND RELATED COMPOUNDS FROM YEAST CELLS DURING DIFFERENT GROWTH CONDITIONS

I. L. Cameron W. D. Fisher[1]

The chromatographic separation methods described by Anderson *et al.*[2] have been applied to cold-acid-soluble extracts of yeast (*Saccharomyces cerevisiae*). Spectrophotometric analysis of the column effluent at 260 and at 280 mμ shows 30 peaks. Comparison of the R_F values and the 260/280 mμ absorbance ratios with those of mixtures of known purine and pyrimidine bases, nucleosides, and nucleotides permits presumptive identification of the nucleotide derivatives from yeast. The following compounds were identified in order of elution from the column: Cyd, Urd, Ura, Hx, Ino, Ade, Guo, Gua, CMP, UMP, IMP, UDP-*N*-acetyl glucosamine, AMP, UDP-glucose, CDP, GMP, UDP, ADP, GDP, UTP, ATP, and GTP.

The nucleotide pool of an adenine- and uracil-requiring strain was analyzed before and after removal of these bases from the synthetic medium (see Fig. 5.6.1). Briefly, the results of removing adenine and uracil showed a decrease in the cellular pool of uracil and its derivatives, uridine, UMP, and UDP, and an increase in the cellular pool of adenine and its derivatives, AMP and ADP (see Table 5.6.1).

Regrowth from stationary phase causes the cellular content of UDP-glucose and UDP-*N*-acetyl glucosamine to drop, which suggests the possible utilization of these compounds in cell wall formation. Most of the other nucleotides show a considerable increase in amount during lag phase. Early in the lag phase the nucleotide

Table 5.6.1. Quantitation of Yeast Nucleotide Pool Compounds

Expressed in millimoles/10^{10} cells

Compound	Grown in MV + Histidine + Adenine + Uracil	Grown in MV + Histidine	Percent Increase or Decrease
Urd	0.249	0.196	−27
Ura	0.374	0.189	−98
Hx[a]	0.112	0.048	−133
Ade	0.489	0.540	+12
UMP	0.080	0.054	−48
AMP	0.290	0.373	+42
GMP	0.173	0.169	
UDP	0.027	0.012	−125
ADP	0.091	0.117	+29

[a]Hypoxanthine.

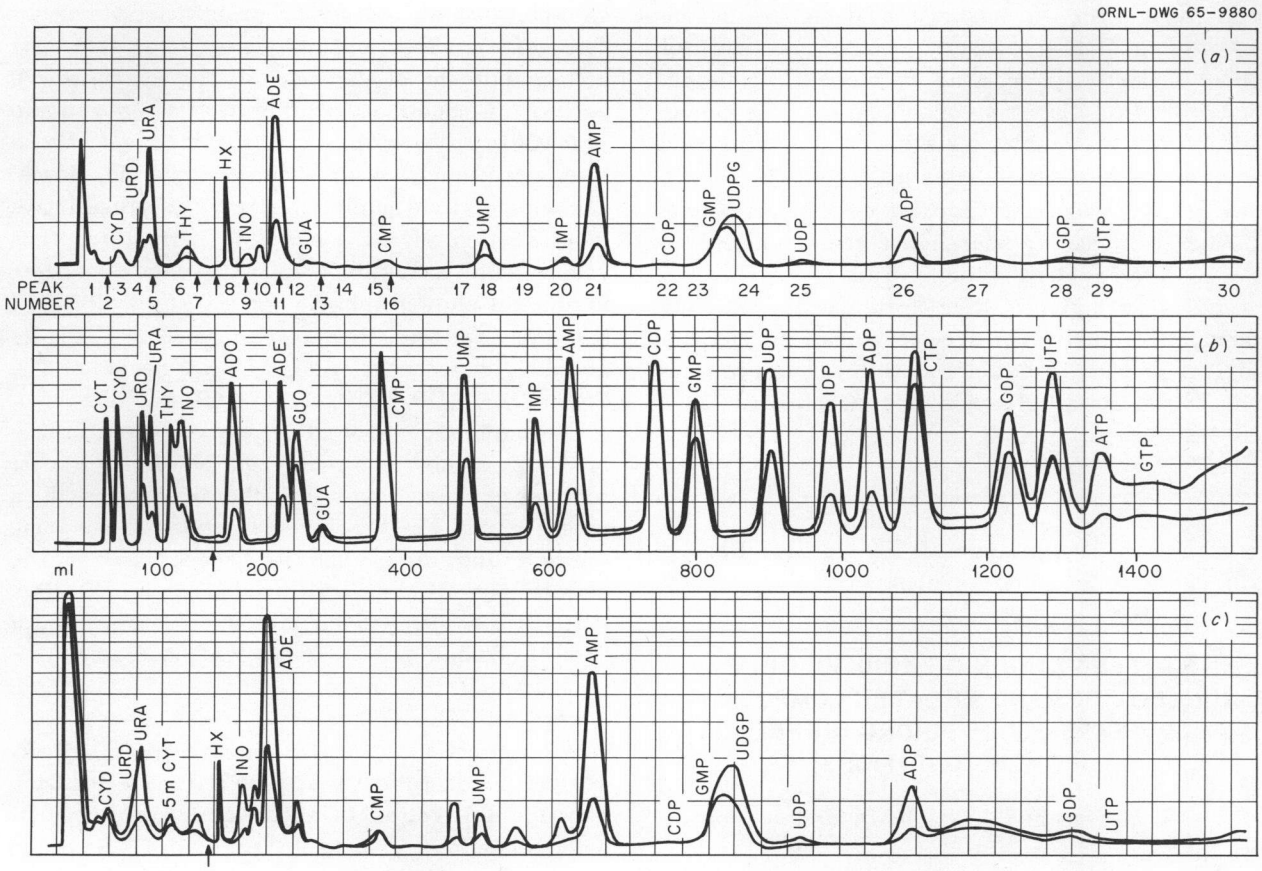

Fig. 5.6.1. (*a*) **Chromatogram of an Ice-Cold 0.5 *M* $HClO_4$ Extract of Lag-Phase Yeast.** The column was eluted with an 1800-ml convex gradient of NaAc at pH 4.4 (0.15 to 3.0 *M*). The absorbance at 280 and 260 mμ using the 1-cm quartz flow cell is shown. The identifiable compounds are indicated. (*b*) **Separation of a Known Mixture of Bases, Nucleotides, and Nucleosides by the Same System.** (*c*) **Separation of the Extracted Nucleotides from Nongrowing Yeast Deprived of Their Required Adenine and Uracil.** Pump flow rate is changed from 0.715 to 1.10 ml/min after 3.5 hr of run.

triphosphates are discernible, and the monophosphates are in low concentration. As the cells first begin to bud, the triphosphates are not observable, while the monophosphates are increased in amount. This suggests that high-energy compounds are increased prior to budding and then used at a time when increased macromolecular synthesis is known to occur (i.e., during budding).

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5.7 MACRONUCLEAR CYTOLOGY OF SYNCHRONIZED *TETRAHYMENA PYRIFORMIS*

I. L. Cameron
G. M. Padilla[1]
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Betty M. Wysinger

Elliott, Kennedy, and Bak,[3] using the electron microscope, have followed cytological changes in macronuclei in *Tetrahymena pyriformis* which were synchronized by the heat-shock method of Scherbaum and Zeuthen.[4] Using Elliott's morphological descriptions, we designed our investigations with the following objectives in mind: (1) to reinvestigate the morphological changes in the macronucleus of *Tetrahymena* synchronized by the

heat-shock method,[4] and (2) to compare these observations with those made with *Tetrahymena* synchronized by a method of temperature synchronization recently reported by Padilla and Cameron.[5] We were then able to relate these findings to the macronuclear cytology of *Tetrahymena* of a logarithmically growing culture. A comparison of cells treated in these three different ways enabled us to evaluate the two different synchronization methods and to gain more information on the structural changes taking place in the macronucleus of *Tetrahymena* as a function of the cell cycle.

Our observations were confined primarily to nucleolar morphology. The results indicate, however, that cells synchronized by the procedure of Padilla and Cameron[5] more closely resemble logarithmically growing *Tetrahymena* in their macronuclear structure than do cells obtained by the Scherbaum and Zeuthen synchronization procedure.

Conclusions. – Both heat shock and stationary-phase conditions cause fusion of nucleoli (see Fig. 5.7.1). In each case the process is reversed when the cell is returned to normal physiological growth conditions. Fusion of nucleoli during the cell cycle of logarithmically growing cells was not observed. Likewise, fusing of nucleoli was not observed when the Padilla and Cameron[5] method of synchronization was used. Thus the macronuclei of cells synchronized by the "one-cold-shock-per-cycle" method[5] more closely resemble macronuclei of log-phase cells than do the macronuclei of cells synchronized by the Scherbaum and Zeuthen[4] heat-shock method.

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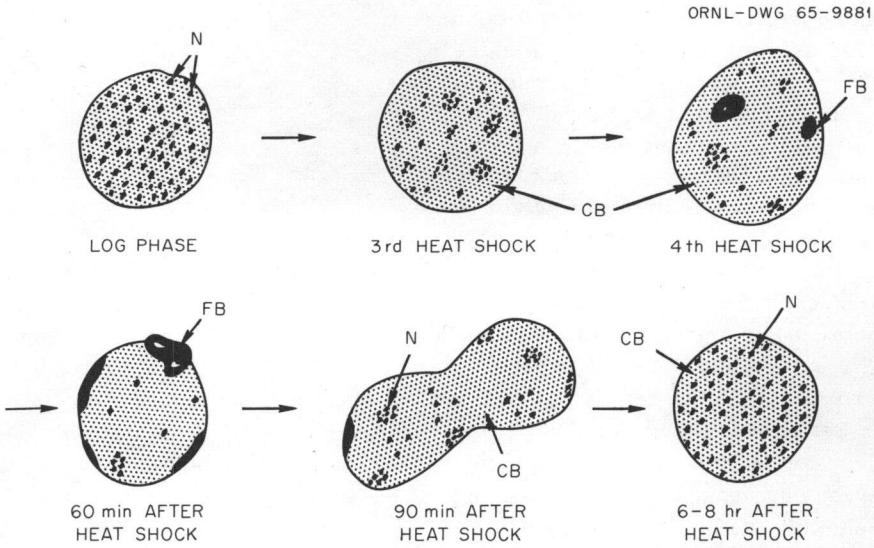

Fig. 5.7.1. Diagrammatic Presentation of Macronuclear Events During Heat-Shock Synchronization of Logarithmically Growing Cell. The last three drawings represent events which occur after the last heat shock. CB = chromatin bodies; N = nucleolus; FB = fusion bodies.

5.8 RELATION BETWEEN THE NUCLEAR DNA CYCLE AND THE DIVISION CYCLE IN MASS CULTURES OF SYNCHRONIZED *TETRAHYMENA PYRIFORMIS* HSM

I. L. Cameron G. M. Padilla[1]

It has been shown that *Tetrahymena pyriformis* strain HSM can be synchronized by a repetitive temperature cycle, that the synchrony can be maintained indefinitely, and that micronuclear DNA synthesis takes place during cytokinesis. Therefore, this synchrony method does not disturb the placement of the micronuclear DNA-synthetic period as compared with logarithmic growth conditions. Apparently, the replication of micronuclear DNA synthesis is intimately coupled to the division cycle in such a manner as to suggest that the cytoplasm and the micronucleus are exerting a regulatory control, one over the other. There is an amicronucleate strain of *T. pyriformis* that undergoes normal cytokinesis and growth. Moreover, the micronucleus in other strains of *T. pyriformis* has never been observed to synthesize DNA, except during cytokinesis. We must conclude, therefore, that the cytoplasm or macronucleus is dominant in its control and that the only course of the micronucleus in this species is to follow this control. The information which controls the micronucleus may indeed be stored in the cytoplasm as a result of some previous macronuclear message.

The macronuclear DNA replication period does not appear to be as intimately locked into the cell cycle as is the micronucleus. One consistent fact is evident from our studies, namely, that although the macronucleus undergoes an amitotic division (without the ordinary condensation of chromosomes as occurs in mitotically dividing cells), the macronucleus never replicates DNA during cytokinesis. This may, therefore, be thought of as macronuclear synchrony in our procedure. Therefore, in our synchrony system, macronuclear DNA replication and cytokinesis are mutually exclusive events in the cell cycle.

In an effort to better understand the relation between the nuclear replication cycle and division cycle in our synchrony regime, we extended our ^{3}H-thymidine labeling studies as follows: We incubated one aliquot of cells in ^{3}H-thymidine from the beginning of the warm period and continued the incubation for 2 hr. Samples were periodically withdrawn for autoradiographic preparation. Two other aliquots were taken 45 min before the beginning of the cold period (at a time when few macronuclei are in DNA synthesis, S phase, Fig. 5.8.1, curves II and III); both these aliquots were also incubated constantly in ^{3}H-thymidine. One aliquot was returned to the cold period at the normal time (curve II, Fig. 5.8.1); the other aliquot was allowed to continue incubation at the warm temperature (curve III, Fig. 5.8.1). Samples were periodically removed from both aliquots and autoradiographs prepared. The autoradiographs were analyzed for the percent of cells showing macronuclear incorporation of ^{3}H-thymidine.

When ^{3}H-thymidine is left in the culture, all the cells that are in or that enter macronuclear DNA synthesis during the incubation period become labeled. The results show that about 50% of the cells are in macronuclear DNA synthesis

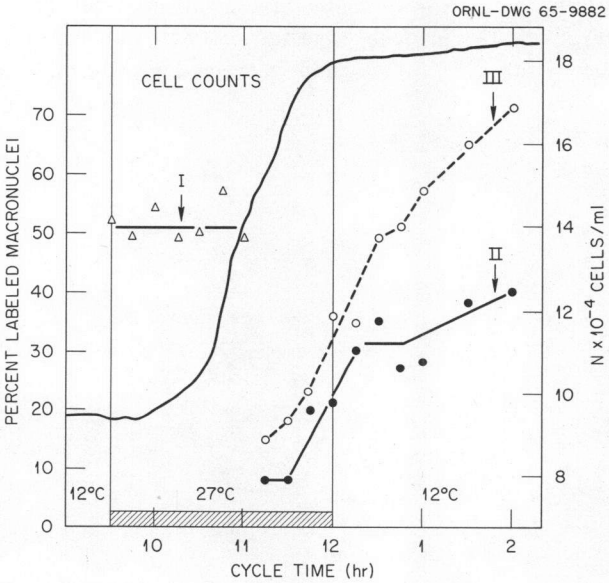

Fig. 5.8.1. Tracing of Continuous Recorded Cell Count of Temperature-Synchronized *Tetrahymena* According to the Technique of James and Anderson (1963). Curves I, II, and III indicate the percent of cells with labeled macronuclei when an aliquot of cells was removed from the parent culture (indicated by the first point of each curve) and exposed continuously to tritiated thymidine. Samples were taken for autoradiography at intervals and the results plotted. In curve II the aliquot was returned to the cold (12°C) at the normal time, whereas in curve III the aliquot remained at 27°C.

at the end of the cold and beginning of the warm period (curve I, Fig. 5.8.1). If other cells had entered macronuclear DNA synthesis during this period, the percentage would have increased. No evident increase in percentage did occur. Therefore, we may conclude that no cells or very few cells enter macronuclear S during the initial 2 hr of the warm period. Earlier work on ^{3}H-thymidine pulsed cells showed that about 1 hr after the cells enter into the warm period the number of cells engaged in macronuclear DNA synthesis begins to decrease, falling from 50% and reaching its lowest level (about 5%) in the next 45 min. Taken together, these findings show that cells in the macronuclear S phase at the beginning of the warm period are either finishing their round of DNA synthesis or can no longer continue synthesis before cytokinesis. The results demonstrate that cells begin to enter macronuclear S during the latter part of the warm period. At the end of the warm period, 18–36% of the cells have initiated macronuclear S (curves II and III, Fig. 5.8.1). The rate of entry of cells into macronuclear S can be determined from the slope of curves II and III from Fig. 5.8.1. Clearly, imposition of the cold period slows the rate of entry into S but does not altogether stop initiation of macronuclear S by other cells. Pulsing with ^{3}H-thymidine during the cold period shows that cells in macronuclear S during the cold period continue DNA synthesis. Indeed, the results suggest that as many as 50% of the cells have finished macronuclear S during the cold period, since no new cell enters macronuclear S during the first part of the warm period. Because no or very few cells divide during the early warm period, we must conclude that even those cells which have finished S during the cold period still require at least 75–90 min of the warm period before they can divide. This suggests that some metabolic and/or morphological events (e.g., stomatogenesis) must occur before cell division begins. It also indicates that the completion of macronuclear DNA synthesis is not the immediate "trigger" for cytokinesis.

References

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Cell Growth and Differentiation

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6.1 MORPHOGENETIC EFFECTS OF DIFFERENT RNA FRACTIONS OF RAT LIVER AND THEIR HYDROLYSATES ON THE NEWT ECTODERM

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Marialuisa Melli[2]

Morphogenetic effects of RNA on the ectoderm of *Triturus* have been studied by a number of workers. Most workers obtained weak neuralizing effects, but Niu[3] claims to have obtained some other specific effects. So far, no one has tried various fractions of RNA for their morphogenetic effects or checked the possibility that degradation products of RNA are playing a role in the so-called induction by RNA.

Biochemical Methods. – The RNA samples were isolated from male albino Sprague-Dawley rats fasted overnight to deplete the liver of glycogen. "Ribosomal RNA" was extracted by phenol from the ribosome fraction of the liver prepared according to Wettstein *et al.*[4] In sucrose-density-gradient analysis the sample showed a small amount of 4S and heterogenous RNA in addition to the 28S and 18S RNA. "Nuclear RNA" was prepared essentially according to Georgiev *et al.*[5] from the nucleus fraction of the liver by a series of phenol extractions. "Soluble RNA" was obtained by the methods of Brunngraber[6] and contained less than 5% DNA as estimated by the diphenylamine reaction. The sample was incubated for 30 min at 37°C at pH 9 to discharge attached amino acids from the sRNA. Sucrose-density-gradient analysis of this sample demonstrated one sharp peak at 4S. A portion of each of the above three RNA samples was hydrolyzed with 0.5 *N* KOH at 37°C for 18 hr. The pH was adjusted to neutral with $HClO_4$, and $KClO_4$ was removed by centrifugation. The RNA samples or their hydrolysates were dissolved in sterile Holtfreter's saline buffered at pH 7.3 with Tris-HCl.

Embryological Methods. – Early gastrula ectoderm of *Triturus pyrrhogaster* or *Triturus vulgaris* was isolated with fine tungsten needles and treated with solutions containing either one of the RNA samples or one of their hydrolysates for 24 hr. No noticeable change occurred in the pH of the test medium before or after the treatment. The explants were further cultured in Holtfreter's saline containing a mixture of penicillin G and streptomycin sulfate (pH 7.3). In control experiments, explants isolated at the same time were

Table 6.1.1. Effects of RNA Fractions and Their Hydrolysates on Differentiation of Isolated Ectoderm

Treatment Medium	Concentrations (μg/ml)							
	50		100		200		400	
	Number of Explants Made	Number of Explants Neuralized	Number of Explants Made	Number of Explants Neuralized	Number of Explants Made	Number of Explants Neuralized	Number of Explants Made	Number of Explants Neuralized
"Nuclear RNA"	27	21	6	2	11	4	7	1
"Nuclear RNA" hydrolysate	24	16						
"Soluble RNA"	27	14	19	11	15	11	23	11
"Soluble RNA" hydrolysate	15	7						
"Ribosomal RNA"	23	0	7	2	6	2	5	0
"Ribosomal RNA" hydrolysate	19	17						

cultured in Holtfreter's saline at the same temperature and pH. The total time interval between isolation and fixation was kept the same for experimental and control explants. Throughout the experiment a sterile condition was maintained.

Results and Discussion. – The results of the histological examination of the explants treated with different samples are summarized in Table 6.1.1. The neural differentiation observed varied from irregular mass of neural tissue to well-formed neural structure of the archencephalic type. The control explants, on the other hand, always developed into the aggregates of epidermal cells. In the RNA series a higher frequency of neural induction was obtained with the "nuclear" and "soluble RNA," while the "ribosomal RNA" indicated very low morphogenetic activity. If the inducing ability of the "nuclear RNA" was due to its complement in the "soluble RNA," we would have expected a stronger effect in the "ribosomal RNA" too, since 4S appeared in both "ribosomal" and "nuclear RNA" sucrose-gradient profiles. It appears unlikely that messenger RNA activity was involved in the present experiment, since in the rat liver it appears to sediment under the 18S RNA peak. Results with "soluble RNA" lend additional support for noninvolvement of messenger RNA activity. Results obtained with the hydrolysates of different RNA's, which should contain various nucleotides and a small amount of nucleosides and bases, demonstrate that neuralization can be caused by low-molecular-weight substances, probably by nucleotides. Rather striking is the fact that the hydrolysates of "ribosomal RNA" caused frequent neuralization, while "ribosomal RNA" did not cause neuralization at the same concentration. In view of those results it appears probable that the neuralizing effect of the "nuclear" and "soluble RNA" was due to nucleotides released from RNA. It is known that the terminal –CCA group of sRNA is very labile and that the adenine nucleotide is very easily released in solution. The small amount of sRNA present in the "ribosomal RNA" represents the RNA transfer bond to the ribosomes during the process of protein synthesis; this sRNA should not release its labile terminal adenine nucleotide.

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6.2 A MEMBRANOUS COMPONENT IN AMPHIBIAN OOCYTE NUCLEOLI

O. L. Miller, Jr.

During the lampbrush-chromosome stage of *Triturus* oocytes, each of the several hundred free peripheral nucleoli exhibits in thin section a compact fibrous core surrounded by a granular cortex. When these nucleoli are isolated in 0.5 to 0.15 *M* saline, the two components remain together. If isolated at a lower molarity, the granular cortex disperses, leaving a coarsely fibrous, less compact core which is known to contain DNA, RNA, and protein.

As the isolated cores gradually separate from the nucleoplasm, a low-contrast material attached to the cores can be observed with the phase-contrast microscope. When only salts are used in the isolation medium, this component generally maintains a spherical shape (2 to 3 μ in diameter) and, when precipitated and positive stained, appears opaque in the electron microscope. When isolated in saline with 0.1 to 0.2 *M* sucrose, this structure becomes attenuated and may extend up to 20 μ as a thin low-contrast filament attached to individual cores. When these filaments are observed with the electron microscope, they appear as flattened membranous tubules ranging from 500 A to 0.5 μ in diameter and containing granules 500 to 600 A in diameter, irregularly spaced within the membranous component.

Although a possible de novo formation of a membrane-like structure from soluble materials after isolation cannot be entirely ruled out, the similarity of the attached structures from core to core and the reproducibility from oocyte to oocyte strongly suggest that each nucleolus in this cell contains a membranous component. The possibility that this component may be involved in the transformation of fibrous materials in the core into the granular component of these nucleoli is suggested.

6.3 A CHROMATOGRAPHIC SYSTEM FOR THE YOLK PROTEINS OF TELEOST EGGS AND A PRELIMINARY ANALYSIS OF THE ISOLATED COMPONENTS

R. A. Wallace

A recently developed chromatographic method[1] has facilitated the resolution, purification, and analysis of egg-yolk proteins of many vertebrate species. This method involved the solvation of yolk granules in 0.01 *M* citric acid–0.06 *M* 2-amino-2-methyl-1-propanol (Monol), application of the solution to a specially prepared TEAE-cellulose column, and elution of the component proteins by a concave gradient into 0.25 *M* citric acid–0.75 *M* Monol. The elution positions of the various proteins appeared to be related to their extent of phosphorylation.

This method has been found to be not applicable to the yolk proteins from the eggs of most teleosts, however. Such eggs contain a fluid rather than granular yolk, and, when applied to a TEAE-cellulose column equilibrated with the usual starting buffer, most of the proteins are not bound by the adsorbent. After trying several different solvent systems, it was found that this difficulty can be remedied simply by diluting the usual starting buffer to a concentration of 2.5 m*M* citric acid–15 m*M* Monol and eluting in the normal manner. No pH front or other anomalous behavior was observed for this procedure. An example of a chromatogram thus obtained for the eggs of the killifish *Fundulus heteroclitus* is provided in Fig. 6.3.1. Two lipoprotein components absorbing at 280 mμ were observed (elution positions 0.18 and 0.27), and unlike the lipoproteins present in the yolk of other vertebrate eggs, they do not appear to be phosphorylated. Furthermore, a nonabsorbing (at 280 mμ) and possibly heterogeneous phosphoprotein was observed at elution position 0.63–0.70. This phosphoprotein was found to be completely acid soluble and hence is similar to the phosvitin recently prepared from the eggs of the brown trout[2] (but in this respect is unlike the amphibian and avian phosvitins).

The modified chromatographic procedure has also been used with success for the yolk proteins of the rainbow trout (*Salmo irideus;* lipoproteins eluted at positions 0.17 and 0.52 and phosvitin at 0.67–0.74) and the toadfish (*Opsanus tau*; lipoproteins eluted at positions 0.29 and 0.43 and

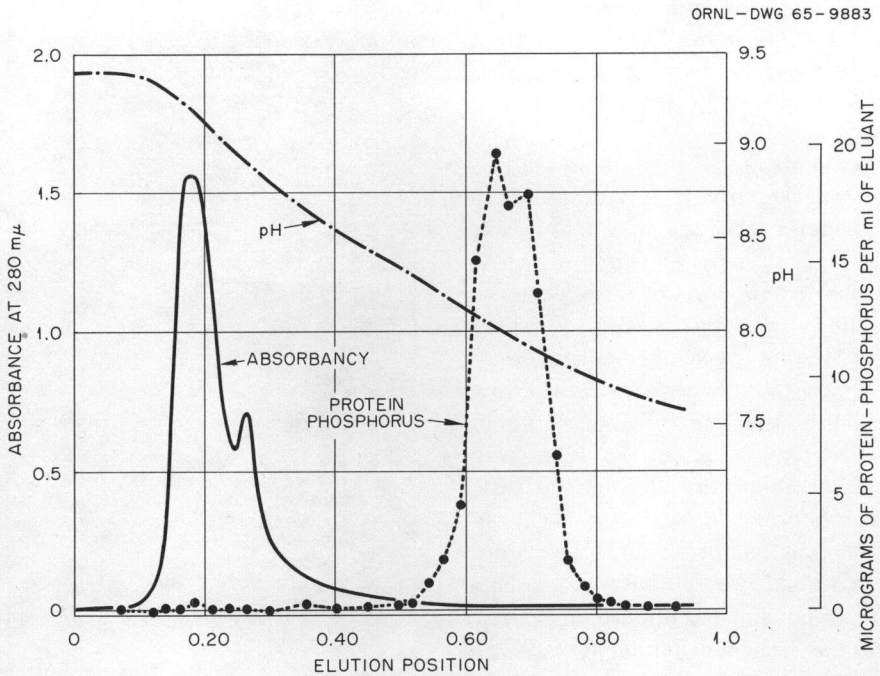

Fig. 6.3.1. Gradient Elution of 270 mg of Yolk Protein (*Fundulus heteroclitus*).

phosvitin at 0.72–0.78). Thus, representatives of three of the orders of Teleostei have been examined.

Further work is now in progress on the physical and chemical characterization of the isolated proteins. Of particular interest is the very low extent of phosphorylation observed for the teleost yolk lipoproteins. It has been suggested[3] that the formation of insoluble yolk granules or crystals in the oocytes of other vertebrate species is a direct result of lipoprotein phosphorylation. The lack of a phosphorylated lipoprotein in teleost eggs and the concomitant existence of a fluid yolk would thus be consistent with this hypothesis. Work is also now in progress to see if a protein-phosphorylating system such as that observed for the amphibian oocyte[3] is present or lacking in the developing teleost oocyte.

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6.4 IMMUNOCHEMICAL COMPARISON OF α-CRYSTALLINS BETWEEN MOUSE AND NEWT

Chinami Takata W. D. Fisher[1]
Tuneo Yamada

Introduction. – As a part of the project on cellular differentiation in the Wolffian lens regenerating system, continuous efforts are being directed toward obtaining a specific antiserum against α-crystallins. All attempts to isolate a pure fraction of α-crystallins from the newt lens have failed, primarily for lack of sufficient lens material for extraction. In this experiment a pure fraction of α-crystallins was isolated from the

mouse lens, an antiserum was prepared against it, and immunochemical tests were done to find out whether the antiserum can be used for detection of newt α-crystallins in our lens regenerating system.

Materials and Methods. – Mouse α-crystallins were obtained by centrifugation in a sucrose gradient in the B-IV zonal centrifuge. A rabbit was immunized with 40 mg of mouse α-crystallins in Freund's adjuvant by injecting intravenously, subcutaneously, and intraperitoneally. Three weeks later the rabbit was given, intraperitoneally, 30 mg of alum-precipitated mouse α-crystallins. After ascertaining that the titer of the antiserum as determined in preliminary precipitin tests was sufficiently high, the rabbit was bled on the tenth day after the last injection.

Results and Discussion. – Table 6.4.1 and Fig. 6.4.1 show the results obtained with immunoelectrophoresis (Veronal buffer, pH 8.6, μ = 0.05, 6 v/cm, 2 hr) and the immunodiffusion test (1.25% agar or agarose in 0.01 *M* phosphate-buffered saline, pH 7.0), using serial dilutions of α-crystallins. The mouse α-crystallins could be shown to be immunologically distinct. In the immunoelectrophoresis of the antiserum and the mouse α-crystallins fraction, only one line appeared at the anode side of the origin. Two more lines appeared at the cathode side of the origin in the immunoelectrophoresis of mouse lens extract, in addition to the above line.

It is known that α-crystallins are not species specific in vertebrates. The data described here suggest that only one component of mouse α-crystallins is common to newt α-crystallins. From other data it appears probable that there are components in the newt α-crystallins which do not react with the present antiserum against mouse α-crystallins.

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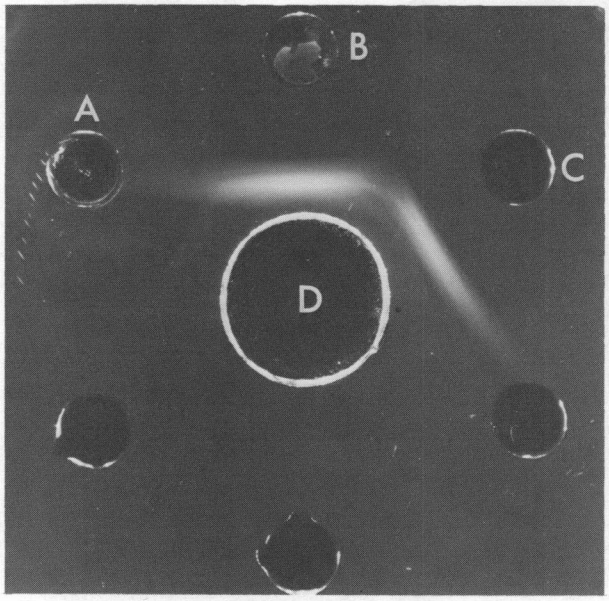

Fig. 6.4.1. Immunodiffusion Pattern with Antiserum Against Mouse α-Crystallins. A = newt lens; B = mouse α-crystallins; C = mouse lens; D = antiserum against mouse α-crystallins.

References

[1]Subcellular and Viral Physiology section.

Table 6.4.1. Results in Immunoelectrophoresis and in the Immunodiffusion Test with Antiserum for Mouse α-Crystallins

Antigens	Immunoelectrophoretic Pattern with Antiserum Against Mouse α-Crystallins	Lines Formed in Immunodiffusion Test[a]
Mouse α-crystallins	o	*a, b, c*
Mouse lens extract	o	*a, b, c, d*
Newt lens extract	o	*a*

[a]Compare Fig. 6.4.1.

6.5 FURTHER STUDIES ON TEMPORAL SEQUENCE OF DNA SYNTHESIS AND TISSUE SPECIFIC PROTEIN SYNTHESIS IN THE LENS REGENERATE

Chinami Takata Tuneo Yamada

Introduction. – In the last report[1] it was indicated that during Wolffian lens regeneration the first appearance of lens specific antigens occurs after final DNA synthesis of regenerate cells and that the time interval between the final S phase and the time of appearance of antigens differs according to the nature of antigens. In the reported experiments applying immunofluorescent autoradiography, the shortest time interval needed for the appearance of fluorescence for a group of antigens after the injection of ^{3}H-thymidine, which led to the labeling of the same cells, was used as a measure for the time interval between the final S phase and antigen synthesis. In the primary lens fiber cells the shortest time interval for γ-crystallins was found to be 2 days, while that for α- and/or β-crystallins was 12 hr. The observation has since been repeated on a larger number of cells. Further, the study was extended to the secondary lens fiber cells, in which some difference in the outcome was expected from earlier immunofluorescent data.

Materials and Methods. – The adult newts *Triturus viridescens* at 13, 14, 15, 18, 20, and 24 days after bilateral lens removal were used in the present study. The immunofluorescent autoradiography procedure used has been described.[1]

Results and Discussion. – *Primary Lens Fiber Cells.* – As shown in Table 6.5.1, coincidence of immunofluorescence for lens specific antigens and silver grains was first observed in 4 in a total of 94 labeled cells at 3 hr after injection. With the increase of the time interval after injection there was a tendency of increase in the frequency of coincidence. Apparent wide variation in the time interval needed for the labeled cells to become fluorescent is caused, at least partly, by the long S phase (15.5 hr) of those cells. The fact that not 100% coincidence was reached even after 24 hr may be partly due to the possibility of labeling in an S phase earlier than the final one in some of the counted labeled cells. The shortest time interval

Table 6.5.1. Coincidence of Immunofluorescence for Lens Specific Antigens and Labeling with ^{3}H-Thymidine in the Primary Lens Fiber Cells

Hours After Injection	Stage Fixed	Number of Regenerates	Number of Fluorescein-Stained Cells with Silver Grains	Number of Cells with Silver Grains
1	IV, V, VI, VII	5	0	41
3	IV, V, VI	6	4	94
4	IV, VI	4	9	42
5	VI	1	2	20
6	V, VI	6	8	91
10	V	2	8	42
12	IV, VI, VII	5	22	97
14	V, VII	2	14	30
16	VI	2	2	3
18	VII	2	10	10
20	VI	1	4	4
24	V, VI, VII	6	24	39
	Total	42	107	513

Table 6.5.2. Coincidence of Immunofluorescence for γ-Crystallins and Labeling with ^{3}H-Thymidine in the Primary Lens Fiber Cells

Time After Injection	Stage Fixed	Number of Regenerates	Number of Fluorescein-Stained Cells with Silver Grains	Number of Cells with Silver Grains
1 hr	IV, VI, VII	4	0	44
3 hr	IV, V, VI, VII, VIII	5	0	67
6 hr	V, VI, VII	5	0	55
12 hr	IV, V, VII	3	0	46
20 hr	VII	1	0	8
1 day	VI, VII, VIII	4	0	29
1.5 days	VI, VIII	4	1	25
2 days	VII, VIII	5	14	38
3 days	VI, VII, VIII	7	24	52
4 days	VI, VII, VIII	4	53	73
6 days	VIII	2	51	51
	Total	44	143	488

needed for appearance of immunofluorescence for γ-crystallins in the cells with silver grains, on the other hand, was 1.5 days (Table 6.5.2). The frequency of the coincidence increased with the time interval after injection and reached 100% in the 6-day series. Since the generation time for those cells is approximately 4 days, the possibility is open that in the later phase of the experiment for γ-crystallins not the final S phase but the one preceding the final one was labeled.

Secondary Lens Fiber Cells. – One hour after the injection the immunofluorescence for lens specific antigens was already detectable in some labeled prospective secondary lens fiber cells which were localized in the lens epithelium and equatorial area of regenerating lens. As to the immunofluorescence for γ-crystallins, none of the labeled prospective secondary lens fiber cells indicated it in the 1-hr series. Coincidence of immunofluorescence for γ-crystallins and silver grains only occurred from 1.5 days after injection.

The data indicate that α- and/or β-crystallins begin to be detected in primary lens fiber cells 3 hr after the last S phase, and in the secondary lens fiber cells probably before the final S phase. γ-Crystallins become detectable both in the primary and secondary lens fiber cells from 1.5 days after the last S phase.

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6.6 IN VITRO FORMATION OF THE LENS IN A CHEMICALLY DEFINED MEDIUM

D. H. Reese Tuneo Yamada

For further cell biological analysis of tissue transformation occurring during Wolffian lens regeneration, a technique is needed which allows regeneration under a defined in vitro condition. Several attempts at in vitro culture of Wolffian lens regeneration made by earlier workers have led to negative results. We were able to find out some

culture conditions under which lens regeneration can be kept in progress. In the present experiment the method was used to demonstrate a difference in the capacity to proceed in vitro between different phases of cellular transformation as defined in earlier reports.[1]

Method. – Bilateral lentectomies were performed on adult *Triturus viridescens*. After periods of 11–12 and 16–17 days from operation, the iris with the regenerate was removed from the left eye for culture. The entire right eye was removed simultaneously and immediately fixed for subsequent histological examination. The regeneration stage of the right eye thus determined served as a basis for judging the progress of regeneration in the left regenerate cultured in vitro. All explants were cultured in a plastic organ-culture dish on lens paper supported by a stainless steel wire grid. Waymouth's MB 752/1 culture medium diluted 2:1 with glass-redistilled water plus streptomycin and penicillin was used. The cultures were maintained at 22 ± 1°C for 8 days with one change of culture media at 4 or 5 days. A gas phase of 5% CO_2 in air was employed during the course of the experiment. After the culture period the tissues were fixed in Bouin mixture and studied in histological sections.

Results and Discussion. – When the explants were classified according to the regeneration stage at the time of explantation and the morphological findings of cultures were summarized, the following results were obtained (Table 6.6.1): (1) Explanted before definite depigmentation of dorsal marginal cells (stage II), some irises produced depigmented cells while the others failed to do so, although depigmentation should have occurred in vivo. (2) Explanted soon after depigmentation of dorsal marginal cells, the irises formed a group of depigmented cells which showed high relative mitotic indexes, comparable to the maximum attained by normal regeneration. Morphology of the cultured regenerate differed from that to be expected in vivo in the total absence of fiber differentiation. In other words, cells failed to enter into the terminal cell cycle as they do in the in vivo condition. The regenerates in vitro can also be distinguished from the normal lens vesicle (stages IV and V) by complete absence of a central cavity. (3) After explantation at the early lens vesicle stage (stage IV), about half the regenerates showed conditions as in (2), while the other regenerates produced a group of fiber cells. (4) Explanted at a later stage, in which cells in the internal layer of lens vesicle have already entered in the terminal cell cycle, all explants produced a group of cells in fiber differentiation, as well as a group of dividing cells similar to that observed in (2). On the basis of comparison with the control stage of the right eye of the same animal, a progress in fiber differentiation during the culture period can be concluded. In most cases the morphology of the lens deviated from the normal in that the cells

Table 6.6.1. Summary of Progress of Differentiation in Isolated Regenerates

Regeneration Stage at Time of Isolation	Total Number of Explants	Regenerates After 8 Days Culture		
		Number That Have No Depigmented Cells	Number That Have Depigmented Cells Only	Number That Have Both Depigmented Cells and Fiber Cells
II	15	4	11	0
III	19	1	18	0
IV	10	1	5	4
V	5	0	0	5
VI	5	0	0	5
VIII	2	0	0	2
Total	56	6	34	16

comparable to lens epithelium cells formed an aggregate which was connected to the naked mass of fiber cells. Only in one exceptional case did the lens epithelium cover the fiber sphere.

From the above results and further details of the study, the following tentative conclusions can be suggested: (1) The present culture condition either fails or is insufficient in promoting the depigmentation processes. (2) It tends to keep the cells in multiplication and to inhibit the cells from entering the terminal cycle. (3) Once the cells have entered the terminal cell cycle they are able to proceed, under the present culture conditions, in the differentiation process characteristic to the cycle.

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6.7 APPLICATION OF EDSTROM'S TECHNIQUE OF MICROELECTROPHORESIS TO THE STUDY OF NUCLEAR RNA OF LENS REGENERATING SYSTEM

Tuneo Yamada

In our study of subcellular events occurring during Wolffian lens regeneration some information has been obtained concerning RNA synthesis with the autoradiographic technique.[1] For understanding the molecular mechanism of tissue transformation, more precise data on RNA are needed. Conventional biochemical techniques are not readily applicable because we are dealing with a minute amount of tissue in which different types of cells with different types of RNA metabolism are localized side by side. An attempt has been made to explore the possibility of applying the technique of "microphoresis," which was developed in the Histological Institute of the University of Gothenburg, to our system. With this technique, 500 $\mu\mu$g of RNA can be analyzed for base composition. The main part of the preliminary study to be reported here was carried out in the laboratory of J.-E. Edström of Gothenburg, Sweden. I acknowledge his active cooperation in this study.

Method. – Nuclei were isolated with a micromanipulator from sections of regenerates of various stages of adult *Triturus viridescens* fixed with Carnoy's mixture. This operation was done either in a thin layer of paraffin oil or in an oil chamber after treating the section with 0.01 *N* acetic acid. In the case of pigmented cells of the early phase of regeneration, as well as of the normal iris, isolation of nuclei was possible but time consuming, and usually a small amount of cytoplasm adhered to the isolated nuclei. In the case of depigmented cells of later stages of regeneration, the operation was not difficult and led to clean separation of nuclei from the cytoplasm. The nuclei isolated in paraffin oil were further treated with glycerol, put in the oil chamber, and then washed with 0.01 *N* acetic acid. A group of *ca.* 25 nuclei was used as a source for each microphoresis. Extraction of RNA from nuclei with ribonuclease, hydrolysis of the extract with 4 *N* HCl, electrophoresis of the hydrolysate on a single

Table 6.7.1. Adenine/Guanine Ratio of Nuclear RNA of Cells at Different Phases of Transformation During Lens Regeneration

Cellular Phase	Histological Origin	Adenine/Guanine Ratio
Early depigmentation	Dorsal iris at stage II	0.797 ; 0.873
Late depigmentation	Dorsal iris at stage III	0.820 ; 0.892
Early multiplication	Lens vesicle at stage IV	0.820
Late multiplication	External layer of lens vesicle at stage V	0.930
End of multiplication	Internal layer of lens vesicle at stage V	0.957
Epithelial	Epithelium at stage X	0.920
Terminal	Fiber area at stage X	1.135

fiber of synthetic silk, photography at 25 mμ of the electrophoretic pattern, and computation of the base ratio were done according to the published method of Edström.[2] A study of the obtained electrophoretic pattern indicated that the area of uridylic acid was contaminated by an unknown ultraviolet-absorbing material. The source of the contamination is now being examined by Dr. Edström. It was, however, possible to obtain valid data on the adenine/guanine ratio from the electrophoretic patterns above. As shown in Table 6.7.1, an increase of the ratio was suggested as the phases of cellular transformation progressed.

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Radiation Microbiology

Alexander Hollaender[a]	
Mutagen Mechanisms – Bacterial Metabolism (7.1)	**Bacterial Genetics (7.2–7.4)**
G. E. Stapleton[a] Annie S. Angel	H. I. Adler Roy Curtiss III C. E. Terry[b] L. J. Charamella Alice A. Hardigree D. E. Axelrod[c] Claire M. Berg[d] Janet Renshaw[e]
[a]Dual assignments. [b]Consultant. [c]Loanee.	[d]ORINS Fellow. [e]Temporary summer employee.

7.1 RADIATION RESISTANCE AND MACROMOLECULAR SYNTHESIS

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

Introduction. – Microscopic observation of slide cultures of irradiated and unirradiated cells of many strains of *Escherichia coli* has led to the conclusion that most strains of this species show an early or immediate inhibition of growth after x irradiation. A few strains which have a mutation at the *lon* locus show radiation inhibition of cell division, with little or no inhibition of growth, and therefore yield long nonseptate cells.[2]

We have been interested for a long time in the pre- and postirradiation conditions which influence the survival and synthetic capacity of the former and much more general class of cells of this species. Preirradiation growth conditions which increase the radioresistance of most strains of *E. coli* increase the RNA content of cells more so than other macromolecules and, more specifically, the types of ribosomes found in cell extracts.[3] Our present investigations attempt to correlate the presence of small ribosomes 30 and 50S to ability to get protein synthesis under way rapidly and, finally, to correlate this with increased colony-forming ability after irradiation.

Results and Discussion. – Recent data indicate that cells grown to stationary phase in the presence of glucose and containing a preponderance of 30 and 50S ribosomes are able to utilize these particulates extremely rapidly upon reincubation into 70 and 100S and larger ribosomal aggregates at a rapid rate. Under similar incubation conditions the radiosensitive cells, which contain mostly 70 and 100S ribosomes, synthesize, for the most part, the smaller 30 and 50S particles.

Figure 7.1.1 compares the rates of synthesis of RNA, DNA, and protein by unirradiated cells of *E. coli* (χ-160), a derivative of K-12. Radioresistant (glucose-broth grown) and radiosensitive (broth grown) stationary-phase cells were reincubated in fresh broth for 90 min at 37°C, and the relative increases in RNA, DNA, and protein were measured colorimetrically in the trichloroacetic acid–insoluble extracts. It is clear from these

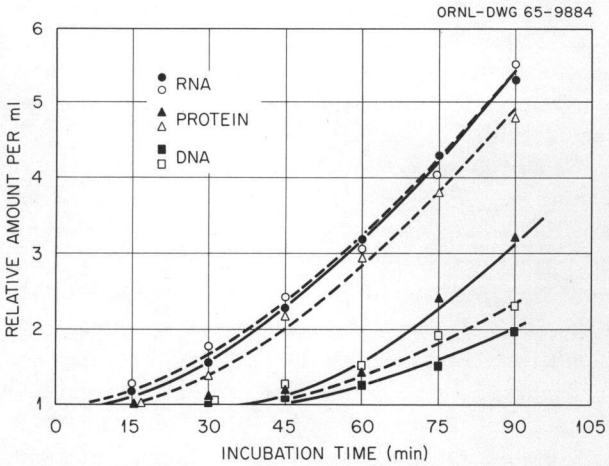

Fig. 7.1.1. Synthesis of RNA, DNA, and Protein by Unirradiated *E. coli* χ-160. Open symbols = resistant cells; closed symbols = sensitive cells.

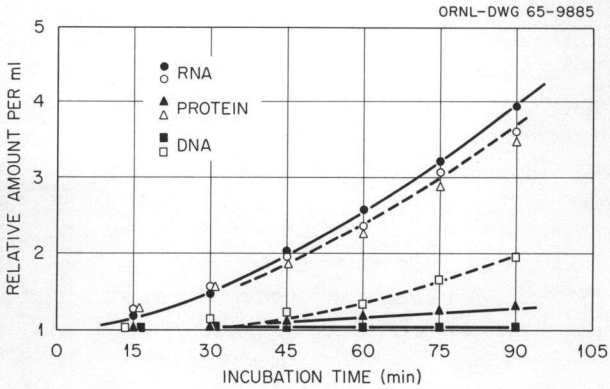

Fig. 7.1.2. Synthesis of RNA, DNA, and Protein by X-Irradiated *E. coli* χ-160. Dose = 20 kilorads. Open symbols = resistant cells; closed symbols = sensitive cells.

data that the relative rates of RNA or DNA synthesis are not much affected by these growth conditions, but protein synthesis is markedly altered. The initial rate of protein synthesis in radioresistant cells parallels that of RNA synthesis, which for these cells is synthesis of 70S and larger ribosomal aggregates. Synthesis of new protein in sensitive cells shows a lag of about 45 min as compared with RNA synthesis, which reflects the synthesis of 30 and 50S particulates assumed to be required for production of new 70S and larger aggregates.

Figure 7.1.2 compares the synthesis of macromolecules by resistant and sensitive cells after 20 kilorads of x rays. (Surviving fractions: resistant cells = 0.5; sensitive cells = 0.025.) Although RNA synthesis is slightly inhibited in both types of cells, protein synthesis in resistant cells again parallels that of RNA synthesis. Synthesis of DNA in resistant cells is inhibited to about the same extent as is RNA synthesis. Protein synthesis and DNA synthesis are drastically inhibited in sensitive cells.

Discussion. – The data reported are completely consistent with those based on microscopic observations in that conditions favorable to postirradiation synthesis are conducive to survival measured by colony formation. It is of some interest that immediate and balanced synthesis of RNA and protein is possible in stationary-phase cultures grown to that stage in glucose broth. This capability seems well correlated with the presence in such cells of large amounts of small 30 and 50S ribosomes.

In regard to radioresistance it is also of interest that cells grown to late lag phase in a complete medium are radioresistant and show a large complement of the smaller ribosomes. Li[4] has recently postulated that late-lag-phase ribosomes are "messenger free" in that they bind either synthetic polynucleotides or streptomycin more effectively than do ribosomes extracted from cells elsewhere in the growth cycle.

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7.2 RECOVERY OF IRRADIATED CELLS PROMOTED BY UNIRRADIATED BACTERIA

H. I. Adler Alice A. Hardigree

Pantoyl lactone and a small number of related compounds promote recovery of irradiated cells of

Escherichia coli.[1] These agents seem to specifically affect the cell division mechanism (cytokinesis) and are only effective on strains that produce long, undivided filaments after exposure to radiation.[2] The concentrations of the division-promoting agents required for maximum effect are very high (approximately 0.1 *M*), and we have suggested that their activity may be due to a trace amount of an unidentified contaminant.

Several years ago Delaporte described a phenomenon that she termed "neighbor restoration."[3] She observed, by microscopic technique, that a dividing cell in an irradiated population of *E. coli* B spread on an agar surface seemed to promote division in neighboring cells, and she postulated that the dividing cell produced a diffusible "division-promoting substance." We were interested in confirming and expanding Delaporte's observation and establishing the relationship between the "division-promoting substance" and the pantoyl lactone group of reagents.

We are using *E. coli* K-12 AB1899NM as a test organism. This strain is streptomycin resistant and forms long filaments after exposure to ionizing or ultraviolet (2537 A) radiation. It is, from a radiobiological point of view, very similar to *E. coli* B.[4] In most experiments a streptomycin-sensitive strain of *E. coli* B/r (ORNL) is used as a donor of the "division-promoting substance." Samples of irradiated populations of AB1899NM are plated in a streptomycin-containing nutrient agar with and without the addition of cells of the donor strain. The donor strain, being sensitive to streptomycin, does not reproduce but does promote the recovery of the irradiated AB1899NM cells. Figures 7.2.1 and 7.2.2 demonstrate this

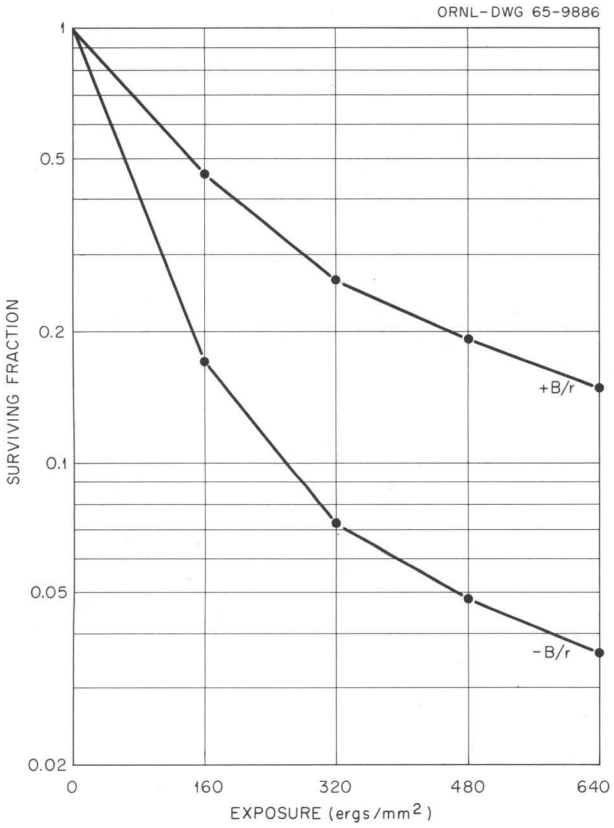

Fig. 7.2.1. Survival of *E. coli* K-12 AB1899 After Exposure to Ultraviolet Radiation (2537 A). Upper curve represents survival on nutrient agar containing streptomycin and approximately 2×10^8 cells of streptomycin-sensitive *E. coli* B/r ORNL per milliliter of medium. Lower curve represents survival on nutrient agar and streptomycin.

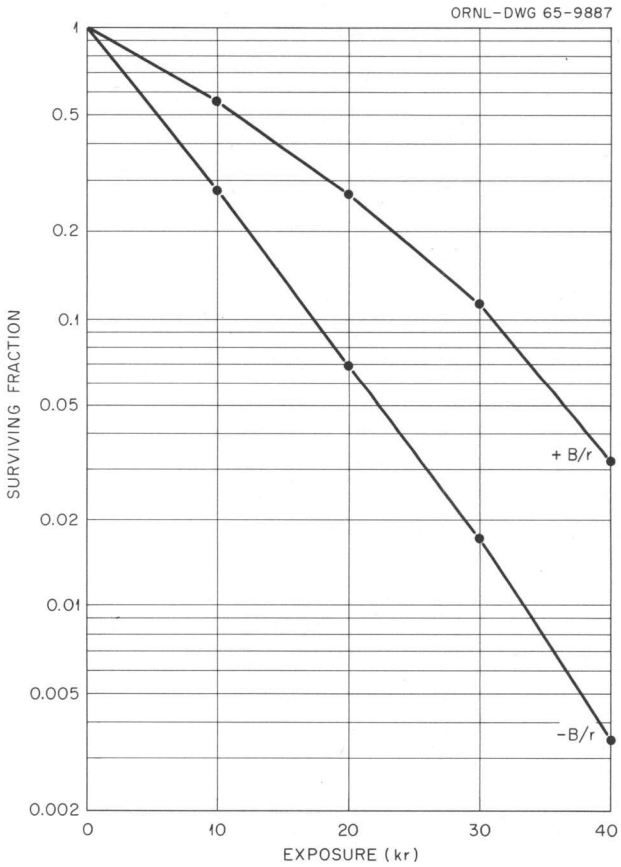

Fig. 7.2.2. Survival of *E. coli* K-12 AB1899 After Exposure to 250-kv X Rays. Plating conditions as for Fig. 7.2.1.

phenomenon for ultraviolet (2537 A) and x-irradiated AB1899NM cells. Several other streptomycin-sensitive strains of *E. coli* have been used as donors, and all are effective. Only filament-forming organisms respond to the "division-promoting substance." Strains such as *E. coli* K-12 P678, which fail to grow after exposure to radiation, are not influenced by the presence of the donor cells. Thus the "division-promoting substance" seems to have the same specificity as pantoyl lactone and related compounds. In order to obtain the maximum recovery effect, donor cells must be added to the plating medium to a final concentration of approximately 2×10^8 cells per milliliter of medium. With this information it is possible to estimate that the "division-promoting substance" produced by donor cells is 200 to 1000 times as effective as pantoyl lactone on a molar basis. Since this substance is a potent radiation recovery agent and apparently is involved in normal cell division, we are currently attempting to isolate and identify it.

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7.3 FERTILITY FACTOR ATTACHMENT IN F^+ STRAINS OF *ESCHERICHIA COLI*

Roy Curtiss III Janet Renshaw

There are two types of F^+ strains in *Escherichia coli* K-12: those which yield stable Hfr mutants (I) and those which seldom if ever do (II). These strain differences were demonstrated by fluctuation tests[1] and by the frequency of recombinants which remained F^- (ref. 2) after interrupted matings. The difference between type I and type II strains is not due to differences in F, the presence of λ, any known genetic marker, or to environment. F transferred from type II strains into an F^- derived by acridine curing of a type I strain gave strains yielding Hfr mutants. The reciprocal strains with chromosomes from type II and F from type I cells have not yielded Hfr mutants. Types I and II F^+ donors gave equal recombinant frequencies in matings with F^- strains. Ultraviolet (uv) radiation stimulated chromosome transfer equally in both types (50- and 100-fold), with maximum recombinant yield being obtained in matings initiated 60 min after uv. Thereafter, recombinant frequency declined, and after 4 hr of post-uv growth, type I cells gave a 20-fold increase in recombinant frequency while type II cells gave pre-uv recombination frequencies (indicating absence of stable Hfr mutants). Time-of-entry experiments for leu^+, ade_3^+, try^+, and all pairwise combinations of these proved that type I and type II strains transferred their chromosomes in linear-oriented manners and not as loops. The results indicate: (1) that two mechanisms exist by which F causes chromosome mobilization, (2) that integration of F to yield Hfr mutants accounts for 5 to 50% of recombinants in crosses with type I F^+ donors and <0.1% with type II F^+ strains, and (3) that the same F can act as an episome in one genetic background (type I) and as a plasmid in another (type II).

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7.4 CHROMOSOME REPLICATION DURING VEGETATIVE GROWTH IN COISOGENIC STRAINS OF *ESCHERICHIA COLI*

Claire M. Berg

Relative gene frequency has been assayed in coisogenic strains of *Escherichia coli* K-12 by P1*kc* transduction to a polyauxotrophic recipient. The Nagata model of chromosome replication[1] suggests that gene frequency in a growing population is a function of the distance of the marker in question from the integrated F factor in Hfr strains. The Jacob-Brenner model[2] predicts that gene frequency in all strains is a function of their oriented distance from a unique chromosomal site.

To test these models, an F^- and various donor-type derivatives of W1485 were employed. The Hfr strains used were: OR 1 (0 lac_{zy} pro_2 ... pro_3 lac_i F), OR 16 (0 *leu*...*met* F), OR 21 (0 pro_3... pro_2 *lac* F), and OR 11 (0 pro_2...ade_3 pro_3 *lac* F). From Hfr OR 11 an F′ (0 ade_3 pro_3 *lac* F) carried over a complementary deletion was obtained. From this F′ strain, two new Hfr's with transposed gene orders were isolated by acridine curing. P1*kc* grown by the confluent plate lysis technique were harvested after $2\frac{1}{2}$ or 6 hr of incubation. Nagata's model predicts altered gene frequencies dependent on the location and polarity of F integration, whereas the Jacob-Brenner model would predict altered frequencies in transposition strains. Relative transductant frequencies were reproducibly indistinguishable among early and late lysates from all eight haploid strains, a result consistent with neither model of chromosome replication. The F′ factor transferred into a nondeletion background yielded a doubled transductant frequency for the duplicated markers, indicating that transductant frequency does indeed reflect gene frequency. With the reservation that transducing phage may not pick up bacterial DNA at random from replicating chromosomes, the data, at present, are not compatible with any model based on a unique origin and/or direction of replication.

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Radiation Immunology

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8.1 CHANGES IN MOUSE SPLEEN RNA AFTER IMMUNIZATION WITH SHEEP RED BLOOD CELLS

R. A. Brown E. H. Perkins
Margaret L. Mann

Introduction. – The intracellular fate and active form of injected antigens are problems which must be resolved before an understanding of the key initial events of antibody formation is possible. Several lines of evidence suggest that formation of a determining site-RNA complex may be one of the initial vital events.

Methods and Results. – The amount of RNA extractable from mouse spleen by phenol doubles within four days after optimum immunization with sheep red blood cells (RBC). In addition to the quantitative change, there are qualitative changes. If one prepares a ribonuclease-resistant fraction of the total RNA and analyzes this fraction on a methylated albumin column, a new component appears within 6 hr after immunization. This new component contains a low-molecular-weight material (<10,000 Dalton's), with a density less than the standard 1.60–1.64 observed for RNA on Cs_2SO_4 gradients.

Discussion. – The data suggest that one has an RNA covalently linked to another moiety. The nature of this complex is under investigation.

8.2 FURTHER STUDIES ON ANTIGEN COMPETITION: QUALITY OF ANTIBODY FORMED

J. F. Albright T. W. Evans

Introduction. – Previous studies on antigen competition[1] led to the conclusion that exposure

of the intact animal to high doses of one antigen (Ag_1) did not completely eliminate the response to a second antigen (Ag_2) when the dose of the latter was sufficiently high to ensure maximum residual response. This result suggested that a fraction of the competent cells existed in a state committed to respond to Ag_2. In order to gain more information on this point, studies were conducted in which the quality of antibody produced in response to Ag_2 administered under competitive conditions was followed. It has been suggested that the production of γM (macro) immunoglobulin is characteristic of the response originating from multipotential (uncommitted) progenitor cells.

Methods and Results. – Twelve-week-old $C31F_1$ mice were injected with 4×10^9 sheep erythrocytes (Ag_1); 16–24 hr later these mice received 4×10^9 rat erythrocytes (Ag_2). Antibody response to Ag_2 was followed by the bleeding of groups of mice each day for a period of ten days. Sera were titrated for total antibody (Ab) directed toward Ag_2, as well as for the portion of antibody surviving treatment with 2-mercaptoethanol (ME). The latter treatment has been shown to destroy the activity of γM antibody but has no effect on γG antibody. At the same time the quality of antibody was studied during normal, noncompetitive response of mice to the same dose of Ag_2. Figure 8.2.1 shows the proportion of the total Ab which was ME resistant (γG globulin) at various times during a normal primary and a competitive primary response to Ag_2. It is clear that the rate at which the proportion of γG globulin increases under competitive conditions is considerably slower than during noncompetitive response.

Discussion. – The interpretation of these results is, at the moment, unclear. It is obvious that either (1) the general notion that γM and γG antibodies characterize responses originating in multipotential and unipotential cells, respectively, or (2) our interpretation – that the residual capacity for response to Ag_2 following high doses of Ag_1 originates from committed (γG globulin–forming) progenitors – is incorrect. A possible alternative, namely, that the residual capacity to respond to Ag_2 originates from multipotential progenitors that are not activated by high doses of Ag_1 owing to autoregulatory factors, is under investigation.

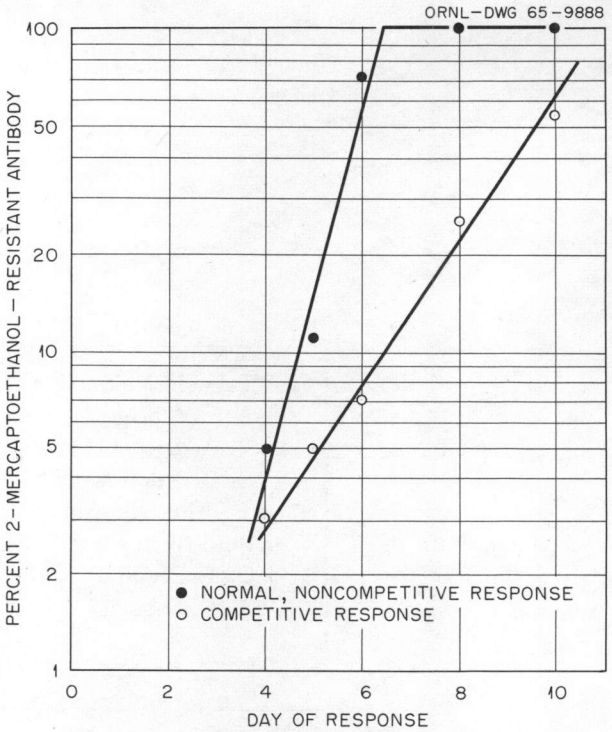

Fig. 8.2.1. Proportion of Total Antibody Which Is Resistant to 2-Mercaptoethanol Treatment at Various Times During Noncompetitive (●) and Competitive (○) Responses to Erythrocyte Antigens.

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8.3 FURTHER STUDIES ON THE FATE OF PLASMA CELLS IN ANTIBODY RESPONSE

Toshikiko Sado[1] Paul Nettesheim
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Introduction. – Previous studies[3] have shown that the serum-antibody-response profile is related to both the sequential change in the population of morphologically distinct immature and mature plasma cells and the change in their metabolic activity. Decrease in the S index of

immature plasma cells and a concomitant increase in the ITT (time required for an immature plasma cell to transform into a mature plasma cell starting from the end of the S phase) indicated to us that a significant number of both immature and mature plasma cells may die during the late phases of antibody response. The present study was carried out to determine if there were plasma cells with atypical nuclei indicative of degenerated cells or cells in the process of degenerating, and if so, the frequency of their occurrence during this period when the metabolism of the plasma cells is changing drastically.

Materials and Results. – A fixed number of primed spleen cells from donor rabbits or mice were cultured in cell-impermeable diffusion chambers, together with an optimum dose of antigen. Spleen cell samples were also obtained from hyperimmunized rabbits. Autoradiographic and immunofluorescent methods were employed. The results showed that during the late phases of antibody response, when the metabolic activity of plasma cells was changing drastically, a significant number of these plasma cells possessed atypical nuclei. There were some cells with bi-, tri-, and tetralobulated nuclei, some with nuclei having one or more budding processes, others with two or more nuclei held together by constricted or broad and diffuse bridges, and still others with fragmented nuclei. These abnormal plasma cells presumably were either degenerated or in the process of degenerating. The frequency of these abnormal plasma cells was constant and relatively high (10 to 20%) when the total number of plasma cells remained constant. Furthermore, 5 to 10% of these abnormal plasma cells were labeled 1 hr after a single injection of ^{3}H-thymidine, indicating that a significant number of them were immature.

Discussion. – These results indicate that, in addition to terminal, mature plasma cells, a significant number of immature plasma cells die during the late phases of antibody response. This would suggest that death among differentiated antibody-forming plasma cells is not solely due to gene-controlled senescence.

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8.4 DIFFERENCES IN THE ANTIBODY-FORMING POTENTIAL OF PRIMED AND UNPRIMED MOUSE SPLEEN CELLS AS REVEALED BY THEIR RECOVERY FROM X IRRADIATION

Paul Nettesheim

Introduction. – Past studies on the effects of physical and chemical agents on antibody response have been based mainly on the use of intact animals. Results thus obtained cannot be taken without reservation, since it has been shown in this laboratory that in the intact animal a regulatory mechanism prevents the lymphoid tissue from responding with its full antibody-forming potential to a given antigenic stimulus. The method of in vivo culture of lymphoid cells, as described previously, has proved to be a valuable tool for quantitative assessment of the kinetics of the immune response. It was therefore used in a comparative study of the recovery of primary and secondary antibody-forming potential after x irradiation.

Methods and Results. – Unprimed and optimally primed (1 ml of 1% rat red blood cells at 8 weeks of age) 12-week-old $C31F_1$ mice were irradiated with 0, 200, 400, and 600 r respectively. At intervals from 1 to 20 weeks thereafter, their spleens were removed and teased; and a fixed number of spleen cells, together with an optimum dose of test antigen, were cultured in 800-r-irradiated isologous recipients. On day 8 after culture, which was shown to be the plateau of antibody response, serum was collected and titrated for hemagglutinins. The results are summarized in Fig. 8.4.1. The primary antibody-forming potential recovers rapidly during the first 4–6 weeks after x irradiation and reaches near-normal levels between 12 and 16 weeks. In comparison, the secondary antibody-forming potential does not recover significantly within the same time period after x irradiation.

Discussion. – These data indicate the existence of at least two distinct types of antigen-stimulable

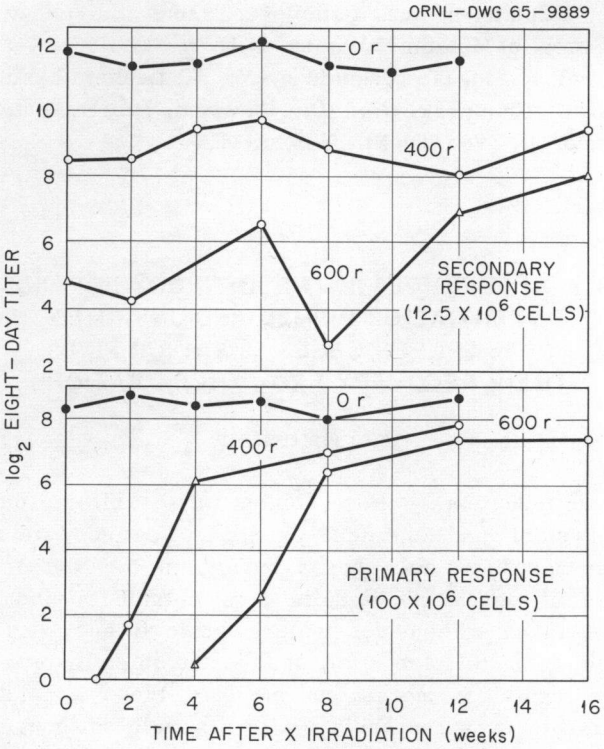

Fig. 8.4.1. Primary and Secondary Antibody Response of Spleen Cells at Varying Time Intervals After X Irradiation.

progenitor cells (PC_1 and PC_2): the compartment of unprimed multipotent PC_1 cells and the compartment of primed unipotent PC_2 cells. The compartment of PC_1 cells recovers after being reduced in size by a physical insult. In contrast, the compartment of PC_2 cells cannot recover in the absence of the test antigen. First results, however, indicate that with increasing priming antigen dose, which probably results in persistence of antigen, the PC_2 compartment can recover. Studies are under way to further establish and elucidate these findings.

8.5 THE SUPPRESSIVE EFFECT OF ULTRAVIOLET IRRADIATION ON FUNCTIONAL ANTIBODY-FORMING CELLS

Toshiteru Morita M. J. Bosma

Introduction. – Previous studies[1] have shown that among functional antibody-forming cells there are some which are quite active in protein metabolism but essentially inactive in RNA and DNA metabolism. These cells are approaching the final terminal state, and they occur with high frequency late in the logarithmic phase of response. It seemed possible that these cells could be useful for analysis of the effects of ultraviolet (uv) irradiation on antibody protein synthesis in the absence of complicating effects on cell division.

Methods and Results. – Primed C31F mice were restimulated with antigen (1 ml of 1% sheep red blood cells). Four days later, their spleens were removed and teased, and spleen cell suspensions were exposed to varying amounts of 2537-A uv irradiation. A fixed number (24×10^6) of irradiated cells were cultured in vivo in isologous irradiated (800 r) mice. At varying intervals after cell transfer, serum samples of recipient mice were collected and titrated for antibody; in addition, the sera were analyzed by agar gel electrophoresis. Photoreactivation experiments were conducted by treating uv-irradiated cells with light of 3500 A at a total dose of 5000 ergs/mm^2. Preliminary experiments indicate that antibody synthesis by spleen cells is quite sensitive to uv irradiation, having a D_{37} dose of about 300 ergs/mm^2. There appeared to be two phases in the uv inactivation of antibody formation, namely, a very sensitive phase and a rather resistant phase. No indication of photoreactivation of uv-damaged cells was found.

Discussion. —In *E. coli* the D_{37} exposure to uv of 180 ergs/mm^2 is characteristic of messenger RNA for β-galactosidase, and 250 ergs/mm^2 is the D_{37} value for general protein synthesis.[2] Our data do not permit a decision concerning the event in antibody synthesis which is characterized by a D_{37} of 300 ergs/mm^2. However, we have largely eliminated the possibly complicating factors of mitosis as the sensitive process.

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8.6 A SIMPLE QUANTITATIVE METHOD TO ASSESS THE ENGULFING AND DEGRADATIVE POTENTIALS OF PHAGOCYTIC CELLS

Toshiteru Morita E. H. Perkins

Introduction. – Since Metchnikoff suggested the importance of phagocytosis in the protection of the host against infection, there has been a continuing search for simple quantitative methods for evaluating phagocytic activity. Most methods fail to yield quantitative information on the sequential events of the phagocytic process, that is, information on the ingestive and degradative capacity of the cells. These parameters are essential to our understanding of the functional potential of phagocytic cells, and their evaluation is the subject of this report.

Methods and Results. – Mouse peritoneal phagocytes were incubated in vitro with opsonized sheep red blood cells (RBC) in 25-ml Erlenmeyer flasks on an oscillating shaker at 37°C. The total volume of the reaction mixture was 6 ml and contained 20×10^6 sheep RBC and 10×10^6 peritoneal exudate cells. At suitable time intervals, 1-ml samples of the reaction mixture were exposed to a brief (40 sec) hypotonic shock which resulted in complete lysis of the extracellular RBC, while peritoneal phagocytes and the intracellular RBC were undamaged. The amount of hemoglobin liberated was a direct measure of the number of unengulfed extracellular RBC. To determine the subsequent rate of intracellular degradation, the peritoneal phagocytes with their intracellular erythrocytes were recultured, and at hourly intervals thereafter, aliquots of this cell suspension were removed and lysed by freezing and thawing, and the amount of intracellular hemoglobin determined.

Hemoglobin determinations were carried out, using Drabkin's reagent.[1] Optical density was read on a Beckman DU spectrophotometer at 420 mμ, the wavelength of maximum absorbance for methemoglobin. Results showed that both extracellular and intracellular RBC decreased exponentially. This exponential decrease can be expressed by the equation

$$N = N_0 e^{-k_i t}, \quad i = 1, 2, \ldots,$$

where k_1 is termed the engulfing index, N_0 the number of extracellular RBC at time zero, and N the number of extracellular RBC at time t. In these experiments, $k_1 = 2.6$, and the $t_{1/2}$ of the extracellular RBC was 16 min. From the same equation, k_2 is termed the degradative index, N_0 the intracellular hemoglobin content of cells at the beginning of reculture, and N the hemoglobin content of cells at time t. The degradative index k_2 provides an important additional parameter for assessing phagocytic function. A $t_{1/2}$ of $1\frac{1}{4}$ hr for the intracellular hemoglobin was a consistent finding.

Discussion. – The distinct advantage of the present method is that one can quantitatively measure the engulfing and degradative capacities of phagocytic cells independently. The method has wide application and should prove to be particularly effective in comparative studies, for example, in the analysis of the effects of physical and chemical agents on the phagocytic process.

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Mammalian Genetics

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Mammalian Cytogenetics (9.3)

Liane B. Russell
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Effects of Radiation on Mammalian Gametogenesis (9.4, 9.5)

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9.1 MUTATION FREQUENCY IN FEMALE MICE EXPOSED TO A SMALL X-RAY DOSE AT HIGH DOSE RATE

W. L. Russell Elizabeth M. Kelly

When the effect of radiation dose rate on mutation frequency in mouse spermatogonia and oocytes was reported,[1] it was suggested that the phenomenon might be due to the occurrence, at low radiation dose rates, of repair of mutational or premutational damage. It was also argued that, if this were the case, then, if the dose were lowered, a level should be reached at which repair would occur even with acute irradiation. This could be the explanation for the results reported here.

Mutation frequency in female mice exposed to a total dose of 50 r of x irradiation at 81 r/min was scored by our standard specific-locus method. Only 3 mutations have been observed in the 68,553 offspring obtained so far. On the basis of the mutation frequency obtained with a dose of 400 r, and assuming a linear relation between mutation frequency and dose, 12 mutations would have been

expected. The observed frequency of 3 is significantly ($P < 0.01$) below this value. There are various pieces of evidence[2] that indicate that most of the mutations involved in these studies are not of the multihit aberration type.

Assuming that these preliminary results will be confirmed and that they can be applied to human hazards, they indicate that the genetic risk from small radiation doses, like that from low dose rates, may be lower than had been estimated on the basis of mutation frequencies from large doses at high dose rates.

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9.2 SKELETAL MUTATION IN OFFSPRING OF IRRADIATED MALE MICE

U. H. Ehling

The difficulty of distinguishing between the effects of newly occurring dominant genetic changes and the within-strain variation has been resolved for a large class of skeletal abnormalities by a method described earlier.[1] The classification used to separate, as far as possible, the existing natural variability from that caused by newly occurring genetic changes is based on the assumption that in the sample size of 3073 mice not more than one mutation would be expected for any particular gene locus, and therefore, that the distinction could best be made by merely dividing the abnormalities according to whether they occurred only once in the whole experiment (class 1), or more than once (class 2). The animals with class 1 abnormalities were subdivided into multiple or single abnormalities. The class 1 single category was further subdivided into abnormalities of the appendicular skeleton and abnormalities of the axial skeleton. With this classification a statistically significant increase of abnormalities in one or several subgroups of class 1 is expected to provide the most sensitive indication of mutations. The data support this expectation. The manifestation of class 2 abnormalities showed no statistically significant dependence on the treatment of the males. A highly significant increase of class 1 abnormalities of the multiple type and an indication of an increase for the bilateral type of the appendicular skeleton were observed in offspring from irradiated males. This suggests that the majority of these anomalies is due to induced dominant mutations.

The present experiment is an extension of an earlier one and was designed to obtain data from progeny selected to sample gametes irradiated in spermatogonial stages. The skeletal abnormalities were scored in offspring of inbred 101 strain male mice irradiated with 0, 600, or 1000 r and mated to inbred C3H strain females. One group of males was exposed to a single dose of 600 r. In the 70 litters produced there were 302 offspring, of which 3 showed a presumed dominant mutation. Another group of males was exposed to a total dose of 600 r in two fractions: 100 r followed by 500 r 24 hr later. In the 83 litters produced there were 277 offspring, of which 5 showed a presumed dominant mutation. A third group of males received two fractions of 500 r separated by 10 weeks. In the 43 litters obtained there were 131 offspring, including 2 with a presumed dominant mutation. In the 506 offspring obtained in 98 litters from control males there was only 1 with presumed dominant mutation. The pooled frequency of presumed dominant mutations from this experiment and earlier experiments[1,2] for 0 r or a 600-r single exposure is summarized in Table 9.2.1.

The ratio between dominant skeletal mutations induced in spermatogonial and postspermatogonial stages is similar to the ratio found in the specific-locus experiments.[3] This result supports the view that a sizable proportion of the skeletal abnormalities may result from gene mutations. If they had been predominantly chromosomal aberrations, one

Table 9.2.1. Pooled Frequency of Presumed Dominant Mutations Affecting the Skeleton

Dose (r)	Stage	Number of F_1 Skeletons	Presumed Mutations	
			Number	Percent
0		1637	1	0.06
600	Postspermatogonial	569	10	1.8
600	Spermatogonial	459	4	0.9

would have expected a larger difference between the frequencies in spermatogonial and postspermatogonial stages. The most significant features of the results, from the point of view of human hazards, are, first, that the frequency of individuals with presumed dominant mutations in offspring of irradiated males is high, even for spermatogonial irradiation, and second, that the majority of these radiation-induced genetic effects on the skeleton are probably deleterious to some degree.

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9.3 THE USE OF X-AUTOSOME TRANSLOCATIONS IN LOCATING THE X-CHROMOSOME INACTIVATION CENTER

Liane B. Russell Clyde S. Montgomery

We suggested earlier that X-chromosome inactivation proceeds from a certain point or region and that its intensity decreases with distance. To obtain further evidence concerning the existence and location of the inactivation center, we have studied five independent translocations between the X and linkage group 1, T(X;1)2R1 through T(X;1)6R1. Autosomal breakpoint mapping, based on 2169 mice, gives the following results (R designates breakpoint): *c*-<0.2-R2-10.4-*p*; R3-0.3-*c*-14.7-*p*; R4-5.3-*c*-19.8-*p*; *c*-22.5-*p*-4.0-R5; *c*-18.1-*p*-7.7-R6. Recombination frequencies of breakpoint with the X-linked marker *Ta* for R2, R3, R5, and R6 are 27.8, 30.9, 4.6–8.3, and 6.0–15.0% respectively (based on 389 mice). In these four translocations the combination R(+)/*Ta* has the +/*Ta* phenotype, indicating the *Ta* locus to be under the influence of the inactivation center. Translocated chromosomes would thus be *Ta*-R2-*tp*-*p* (reciprocal, *c*-R2); *Ta*-R3-*c*-*p*; *c*-*p*-R5-*Ta*; *c*-*p*-R6-*Ta*. The X-inactivation center could be on either side of *Ta*, but its left and right limits are given by the R5-*Ta* and the *Ta*-R2 intervals respectively (arbitrary left-right designations are used here since centromere positions are unknown). The present results again confirm that this center is not the centromere and that "flow" of inactivation proceeds in both directions. The decreasing effect with distance is illustrated clearly. Thus, for R5 and R6, recombination *p*-*Ta* is, respectively, 8.6–12.3% and 13.7–22.7%; average coat areas showing the recessive phenotype are, respectively, 50.3 and 24.5% for *p*, 38.1 and 12.5% for *c*.

Mapping of breakpoints in relation to another X-linked marker is setting finer limits on the location of the inactivation center.

9.4 ^{3}H-URIDINE LABELING OF MOUSE OOCYTES

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

The marked changes in nuclear structure and relationship with the follicular cells which characterize oocyte development also should be associated with alterations in oocyte metabolism. This expectation is supported by variations in radiation sensitivity to both cell killing and mutation production.[1]

Studies on metabolism of developing oocytes, however, have been limited primarily to RNA synthesis in Amphibia, as revealed by ^{3}H-uridine labeling. The label first appears over the nucleolus, then over the entire nucleus, and finally in the cytoplasm, where it remains for long periods of time.[2] Incorporation occurs in growing oocytes and ceases prior to ovulation. This RNA metabolism is primarily one of ribosome storage in preparation for early cleavage of the zygote.[2] Since this process should occur in mammals also, it was hoped that ^{3}H-uridine labeling would reveal the relationships between the follicular stage, metabolic activity, and radiation response of the mouse oocyte.

Young adult female mice were given an intraperitoneal injection of 75 μc of ^{3}H-uridine and killed at intervals of 15 min to 35 days after injection. Ovaries were fixed in Carnoy's mixture, embedded in paraffin, sectioned serially at 3 μ, coated with NTB_2 liquid emulsion, and exposed for 6–8 weeks. Slides were stained with hematoxylin and eosin after development.

As in Amphibia, the label appeared first over the nucleolus, then moved to the nucleus and, subsequently, to the cytoplasm. Also, as in Amphibia,

labeling was limited to oocytes in young and growing follicles. No ^{3}H-uridine incorporation by oocytes occurred in follicles with an antrum. Labeling was definite by 15 min, but became heavier by 30 min after injection. Localization of the label was primarily nuclear until 12 hr, when significant cytoplasmic labeling was first observed. Nuclei and many nucleoli still were labeled at nine days. The large pool of ^{3}H-uridine in the ovarian tissue, permitting continued uptake by oocytes, is apparently the explanation of this prolonged oocyte labeling.

Exact timing of follicular development has not yet been realized in the mouse, but follicles with an antrum can be related to the first few ovulations following a specific treatment, as, for example, radiation. Thus the oocytes in late follicle stages, where no ^{3}H-uridine incorporation occurs, can be assumed to be the same stages which give a high radiation-induced mutation rate as observed by Russell and Kelly.[1] The stages with low radiation sensitivity to mutation induction[1] correspond to those with active RNA synthesis. Thus, at the present time, there appears to be a close agreement between sensitivity to x-ray-induced mutations and RNA metabolism. However, even if this correlation should prove to be exact, ^{3}H-uridine incorporation is only an indicator of general metabolic activity of the oocyte and does not constitute evidence for a direct relationship between RNA metabolism and sensitivity to mutation induction.

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9.5 STANDARDIZATION OF TECHNIQUES FOR KARYOTYPE ANALYSIS IN LABORATORY STRAINS OF MICE

R. B. Cumming

Experiments are in progress which are designed to determine optimum techniques for karyotype analysis in various laboratory strains of mice. These investigations cover both peripheral blood techniques and cultured biopsies.

Several techniques for culturing leucocytes from small volumes of mammalian peripheral blood have been published. These are usually very specific about conditions which are required for success, but there is considerable difference in these specified conditions from one report to the next. Thus, for example, Edwards[1] specifies phytohemagglutinin P (PHA-P, Difco), while Arakaki and Sparkes[2] specify phytohemagglutinin M (PHA-M, Difco). There are also differences in techniques with regard to medium, blood volume, timing, fixation, and harvest. In our own experiments each of these factors is being systematically varied with blood from several strains of mice which are most used for genetic investigations in the Mammalian Genetics section. Slides are scored on the basis of the percentage of cells which yield usable metaphase figures. The quantitative data are at this time incomplete.

A similar and parallel series of experiments are also in progress, using cultured biopsy material from fetal and adult mice. Data from these experiments are also incomplete at the present time.

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Human Cytogenetics

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10.1 THE CHROMOSOMES OF SOME NATIVE WILD RODENTS

P. Carolyn Gooch P. B. Dunaway[1]

A survey of the somatic chromosomes of wild rodents native to the Oak Ridge area has been initiated. The animals studied thus far are the Cotton Rat, the White-Footed Mouse, and the Pine Mouse. Chromosomal observations on the spermatogonia and spermatocytes of subspecies of the Cotton Rat and the White-Footed Mouse different from those reported here have been made by several authors.[2–6] There have been no reports on the chromosomes of the Pine Mouse.

The animals were captured and housed by the Ecology section of the Health Physics Division, and all handling of the animals prior to slide preparation of the fixed cells was carried out in the Ecology laboratories. The method used for obtaining chromosome preparations was a modification of the bone marrow technique of Ford and Hamerton.[7] Considerable difficulty was encountered in finding the optimum hypotonic treatment for the bone marrow cells of these species.

The number of animals studied thus far is small. Chromosome counts and preliminary karyotype analysis on the Cotton Rat and White-Footed Mouse and a tentative chromosome count on the Pine Mouse can, however, be given.

Chromosome counts from spermatogonia and spermatocytes have been reported for the Cotton Rat, *Sigmodon hispidus texianus*, by Cross, and for an unidentified subspecies of *Sigmodon hispidus* by Matthey.[2–6] The chromosome number of 54 was reported in both cases. The Cotton Rats used here are of the subspecies *komareki*. The chromosome number we found for this subspecies, unlike that of the other reported subspecies, is 52. All the autosomal pairs appear to be acrocentric except for one very small metracentric pair. While the size range is quite wide, identification of specific autosomal pairs would be impossible except for one or two of the largest and smallest pairs. The sex chromosomes are the most easily identifiable chromosomes of the complement. The X chromosome is a large subterminal, and the Y is a medium-sized metacentric. They are, in fact, the only chromosomes of these types in the complement.

Two subspecies of the White-Footed Mouse, *Peromyscus leucopus texanus* and *Peromyscus leucopus noveboracensis*, have been reported by

Cross to have 48 chromosomes.[3] The count of 48 for *texanus* was confirmed by Makino.[4,5] The animals reported here are of the subspecies *leucopus*. The chromosome number of this subspecies appears also to be 48. The karyotype consists of several distinguishable pairs and groups of pairs, with the sex chromosomes tentatively identified as a "mismatched" subterminal pair.

We have only a tentative chromosomal count for the Pine Mouse (*Microtus pinetorum*). The number is 62, of which all except two pairs appear to be acrocentric. We plan to continue observations on these and other species.

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10.2 KINETICS OF CHROMATID ABERRATIONS INDUCED IN CHINESE HAMSTER CELLS BY TRITIATED THYMIDINE

J. G. Brewen Gregorio Olivieri

The use of tritiated thymidine in studying cell systems has become very widespread in recent years. Plaut[1] originally cautioned that any interpretations of phenomena observed when tritium was utilized might be subject to some criticism due to the endogenous radiation of the tritium. That the low-energy beta radiation derived from the disintegration of the tritium molecule can cause cellular damage is well documented.

The induction of chromosomal aberrations by tritiated thymidine has been studied. Unfortunately, anaphase figures were scored in all but one of the experiments reported, and in the one case where metaphase figures were scored, only two dose points were used. Both of these facts make a kinetic analysis of the aberrations very difficult. The present experiments were performed in an attempt to determine the yields and kinetics of tritium-induced chromosomal aberrations in mammals.

Several experiments were performed to determine (1) the relation between dose and aberration frequency, (2) the relation between dose and amount of mitotic delay, and (3) the time in the cell cycle at which the mitotic delay occurs. Partially synchronized mitotic cells of a clonal derivative of a Chinese hamster cell line were pulse labeled with differing concentrations of tritiated thymidine when the great proportion of the cells were in the S phase of the cell cycle. Metaphase cells were collected with colchicine at the time of the mitotic peak in some of the cultures. These cells were used to assay chromosomal damage and the amount of radioactive precursor incorporated into the chromosomes. In other cultures, the rate of mitotic activity was measured in order to ascertain how much, if any, mitotic delay occurred at the different tritium concentrations. In still another group of cultures, the cells were pulse labeled with ^{14}C-thymidine at various times to determine when DNA synthesis ended.

The results from these experiments can be summarized very briefly. Both the control and tritium-treated cultures stopped incorporating ^{14}C-thymidine into their DNA at the same time, indicating that there was no delay induced in the S stage by the tritium. The time at which the peak of mitosis occurred was linearly related to the amount of tritium incorporated; that is, the amount of mitotic delay appeared to be linearly related to dose. This observation, coupled with the first observation, indicates that the delay is induced in the post-DNA-synthetic (G_2) phase of the cell cycle. When the yields of the various classes of aberrations were plotted against grain counts, which are a measure of dose, and the curves analyzed by the least-squares method, it was found that the best fit for terminal deletions and exchanges was to a mixed quadratic model ($Y = b_0 + b_1D + b_2D^2$), and the best fit for isochromatid deletions was to a linear model ($Y = b_0 + b_1D$).

The classical views of radiation-induced aberration production state that, when chronic irradiations of the type employed in the present experiments are used, aberrations that have both a one- and two-hit component in their acute-radiation dose curves should have almost linear kinetics. Our observations are not in accord with these views. It is possible, however, to construct a model, taking mitotic delay and stage sensitivity into account, that satisfactorily explains our data. Since G_2 is many times more sensitive

than S to ionizing radiations and since those cells that received the higher doses of radiation from the tritium spent a longer time in the G_2 than the cells that received the lower doses, it becomes clear that the shape of the dose curves can be influenced by these factors. The model accounts for these variations, and when the data are plotted against dose, taking these factors into account, linear curves are obtained for all classes of aberrations.

These experiments tend to point out the complexities and difficulties that stage sensitivity and mitotic delay can introduce into an analysis of radiation-induced chromosomal damage.

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10.3 FINE STRUCTURE OF THE CENTROMERE AND EFFECTS OF CYSTEINE HYDROCHLORIDE ON THE ISOLATED C-METAPHASE CHROMOSOMES

Hiroshi Yamamoto

Introduction. – Previously, some aspects were reported[1] of the fine structure and chemical architecture of isolated chromosomes after tryptic digestion. In the present study the fine structure of the centromere and the effects of cysteine hydrochloride are described.

Methods and Results. – In addition to the acetic acid method[2] for the isolation of chromosomes, isolation by means of cysteine hydrochloride was attempted. After 6 hr of treatment of Chinese hamster fibroblasts with colchicine, the medium in the culture bottles was removed, and the cells were resuspended in 20 ml of distilled water for 10 min as a hypotonic treatment and then centrifuged. The supernatant was removed, and the cell pellets were suspended in 6 ml of 0.005 g/ml cysteine hydrochloride at 4°C. After recentrifugation for 4 min, the pellets of stripped nuclei and isolated chromosomes were resuspended in 1 ml of supernatant and stored at 4°C. Immediately after isolation with cysteine hydrochloride, examination with the phase-contrast microscope showed condensed rod-shaped chromatids, the same as those obtained by the acetic acid method. During a longer incubation period (one to seven days) with cysteine at 4°C, each chromosome gradually became elongated and swollen but never dissolved, and half-chromatid- and quarter-chromatid-sized strands could be recognized as linear continuities. In addition to these results, some of the chromosomes, especially the medium-sized metacentrics, showed a centromeric chromomere pair. The uncoiling of the isolated chromosomes was quite variable. Some of the chromosomes were stretched and swollen; some were more compact. Electron microscopically, some of the chromosomes showed the same type of centromeric region as that reported by Lima-de-Faria.[3] The centromeric chromomeres, or the median zone, were of about the diameter of the half chromatid, composed of convoluted strands 300–600 A in size. In the exterior zone, a pair of twisted quarter-chromatid strands could be seen. The spiralized eighth-chromatid strands 300–600 A in size were rather extended as compared to the median zone.

Discussion. – It has been generally believed that the histones of chromosomes contain neither tryptophan nor cysteine, but recently the idea of covalent linking of DNA molecules by residual proteins containing disulfide cross-links was reported.[4] My micrographs of the isolated chromosomes treated with cysteine hydrochloride do not support this idea, because of the linear continuities of chromosomes after long-term incubation in cysteine hydrochloride.

The fundamental pattern of the centromere as seen with the light microscope has been reported by many authors. Light-microscope studies cannot reveal the details of centromere structure, however, because they are below the limit of resolution. My photograph may be the first demonstration with electron microscopy of the fundamental centromere pattern reported by Lima-de-Faria. The kinetochore structure as seen with the electron microscope reported by Nebel and Coulon[5] is based on an interpretation of centromere structure different from that of Lima-de-Faria.

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AEC-NASA Space Biology Program

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11.1 MAMMALIAN STUDIES WITH 35- AND 57-Mev PROTONS IN THE OAK RIDGE ISOCHRONOUS CYCLOTRON[1]

E. B. Darden, Jr. R. S. Bender[2]

Introduction. – With the completion of the Biology Facility at the Oak Ridge Isochronous Cyclotron, proton irradiation experiments with mice were initiated there in the fall of 1964, as planned.[3] Initially, protons with energies above 35 Mev were not produced in the ORIC, so we confined our first studies to effects on the mouse lens (see below) because of the relatively short range of these protons in tissue. Since protons of energies up to 57 Mev have subsequently become available there, studies have been made to explore the use of 57-Mev protons for whole-body irradiation of mice, as well as to investigate their effects on the lens (see below).

Results and Discussion. – Because cyclotron time is at a premium, we began irradiating mice in groups of four or more in a broad defocused proton beam ($6\frac{1}{2}$ to 7 in. in diameter). With these conditions one needs particularly to know the variation in intensity across the effective area of the beam. In the early experiments, semiquantitative means such as observation of phosphor screens and densimetric analysis of sheet film placed in the beam were used for estimation of cross-sectional uniformity, supplemented by measurements with small (1 × 6 mm) fluororod dosimeters distributed in the beam.

We soon found that a more sensitive, as well as faster, method was required to detect beam inhomogeneities when setting up for irradiation of animals. Accordingly, we have built and tested a remotely operating scanning device consisting of a pair of thimble-type ion chambers which can be positioned in at least 12 preset locations within the effective area of the beam (Fig. 11.1.1). The readouts of these instruments (Radocon dosimeters) give a measure of both rate and integral output. Experience has shown that the only change in

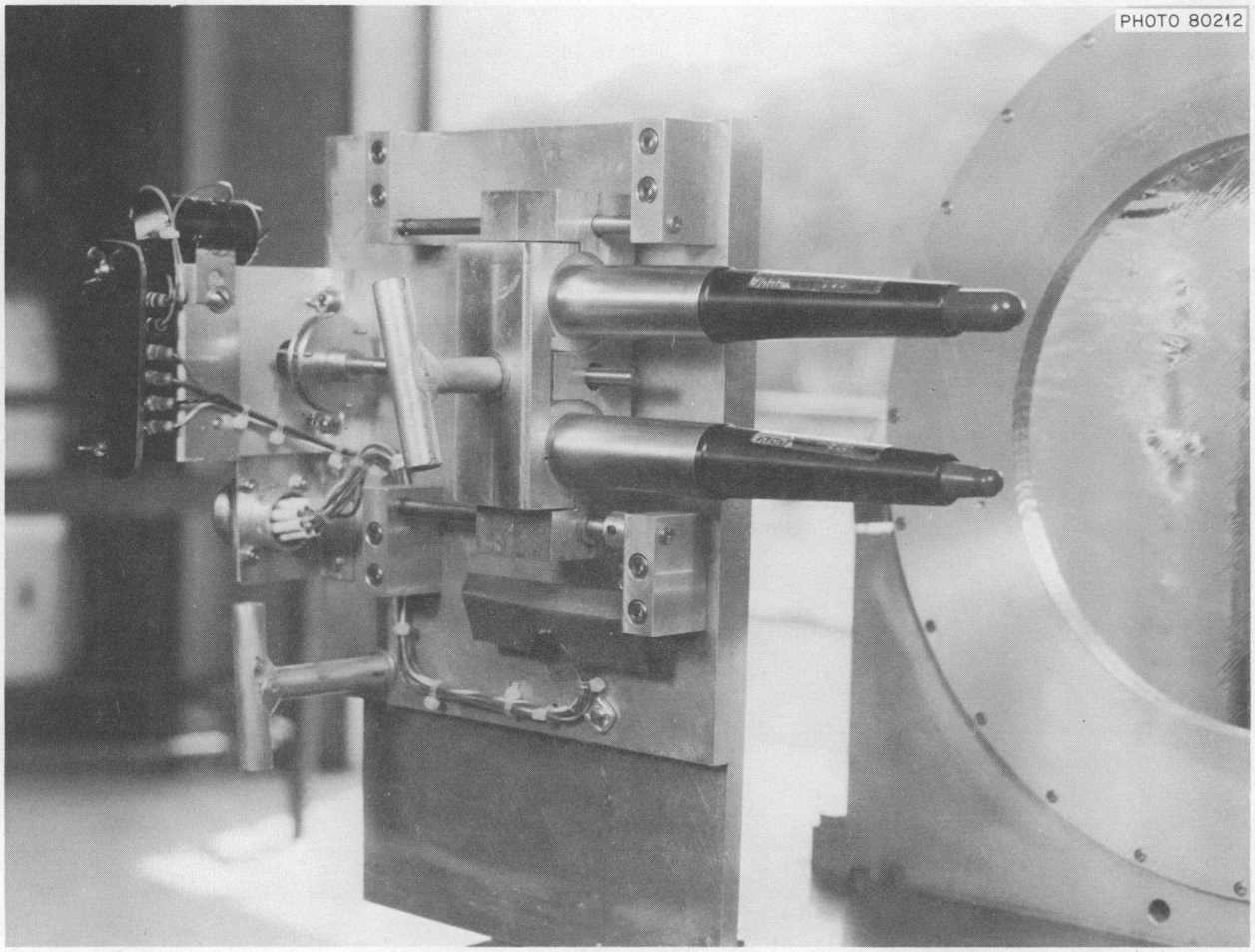

Fig. 11.1.1. Scanning Device for Mapping Cross-Sectional Intensity of Proton Beam. Ion chambers are in exposure position (beam from left). Part of front window of beam dump visible behind chambers on right.

beam properties likely to occur unnoticed during an exposure is an alteration in the field of the positioning magnets, tending to change the effective area of the beam and thus to produce nonuniform exposure. Hence, when beam mapping has been completed, we can move these chambers to opposite positions just inside the beam and effectively monitor it during subsequent exposures.

Protons of 57-Mev energy are sufficiently energetic to traverse the body laterally in young (8-week-old) mice, according to range-energy considerations and dose measurements with small (1 × 6 mm) glass fluororods in phantoms. Although calculations and measurements indicated that radiation to tissue on the exit side of the beam would have LET values 300% or more greater than that to tissue near the entry side, the variation in average LET was reducible to a few percent by rotating the mouse body on its axis; that is, fluororod measurements at depths of 1 to 12 mm in a 24-mm-diam phantom irradiated in this manner agreed within ±3%. Accordingly, for whole-body irradiation, a drive mechanism has been built which permits up to four mice (restrained in thin-walled centrifuge tubes) to be rotated simultaneously in the exposure field (Fig. 11.1.2).

As a result of the satisfactory outcome of the preparatory studies described above, whole-body

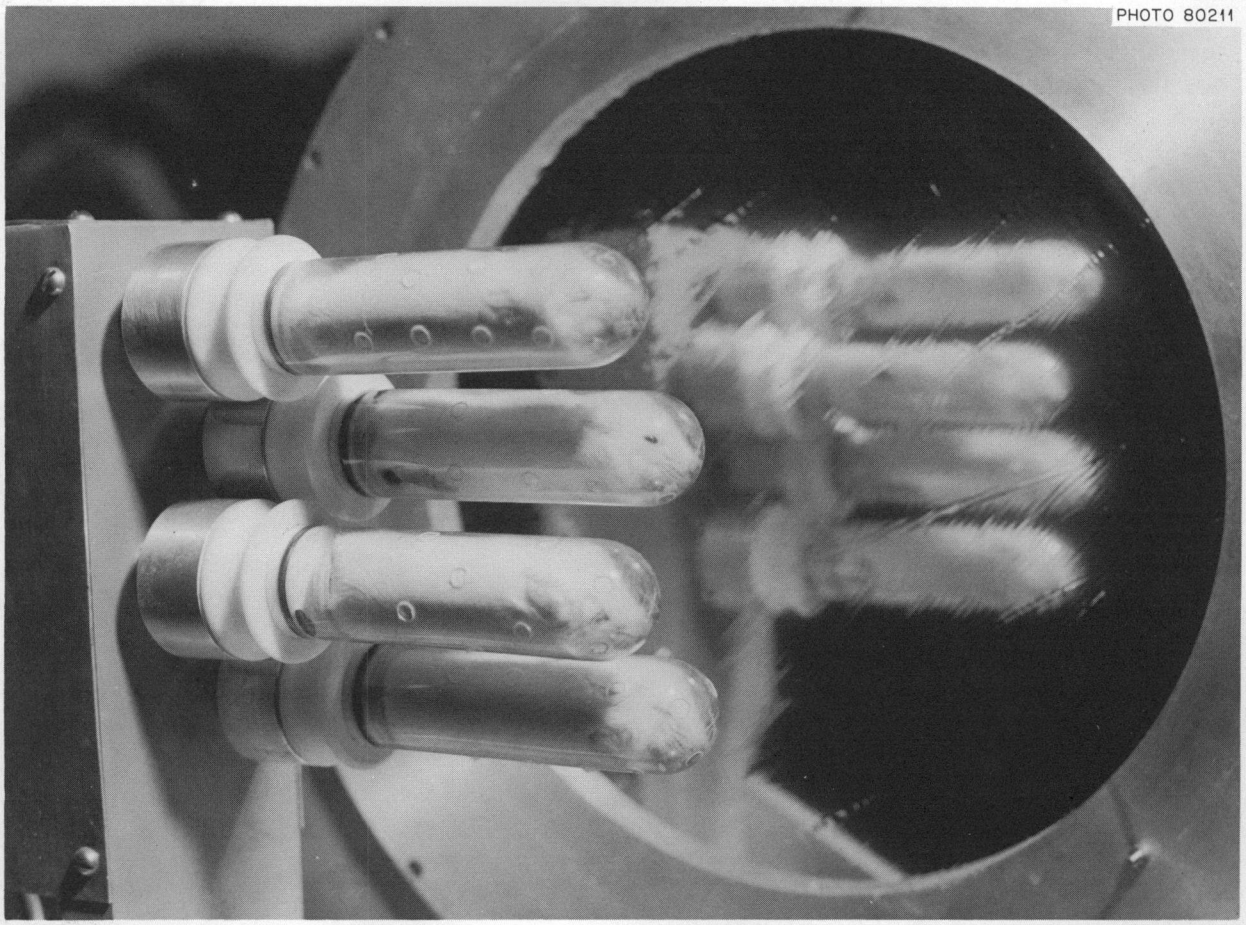

Fig. 11.1.2. Mice in Exposure Position on Rotator (Beam from Left). Mylar window of beam dump in background.

irradiation experiments to study the RBE of 57-Mev protons for life shortening, leukemogenesis, carcinogenesis, and other delayed somatic effects in mice, as outlined previously,[3] are scheduled to begin in the immediate future.

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11.2 PRELIMINARY STUDIES OF LENS OPACIFICATION IN MICE FOLLOWING EXPOSURE TO 35- AND 57-Mev PROTONS[1]

E. B. Darden, Jr. K. W. Christenberry
J. W. Conklin

Introduction. – To determine the RBE of protons of various energies for induction of cataracts, 10-week-old RF/Un female mice were irradiated in the Oak Ridge Isochronous Cyclotron with 35-Mev protons, and resulting opacities of their lenses were assayed as described previously.[2] Since then, experiments have been undertaken with 57-Mev protons, in which anesthetized mice of

similar age were irradiated head on so that both eyes received a single absorbed dose of 0–2000 rads.

Results and Discussion. – Results with 35-Mev protons (Fig. 11.2.1) resemble those with x rays (Fig. 11.2.2) in showing a rapid rise with time in lens opacification, which is dose dependent during the first 50–200 days. Results to date with 57-Mev protons (observations to 150 days) are similar to those with 35-Mev protons. From these preliminary results we can tentatively infer that the RBE of 35- to 57-Mev protons for the induction of cataract in the mouse is not greatly different from that of x rays (Fig. 11.2.3).

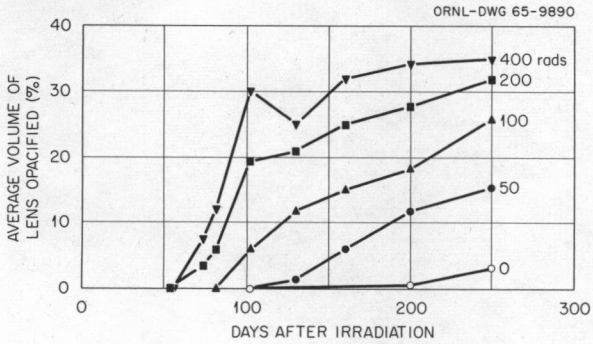

Fig. 11.2.1. Average Severity of Opacification of the Lens in Relation to Time After Irradiation of the Eye with 35-Mev Protons.

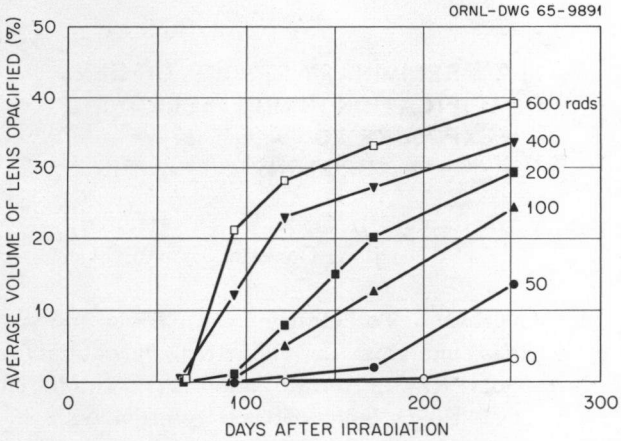

Fig. 11.2.2. Average Severity of Opacification of the Lens in Relation to Time After Irradiation of the Head with 300-kev X Rays.

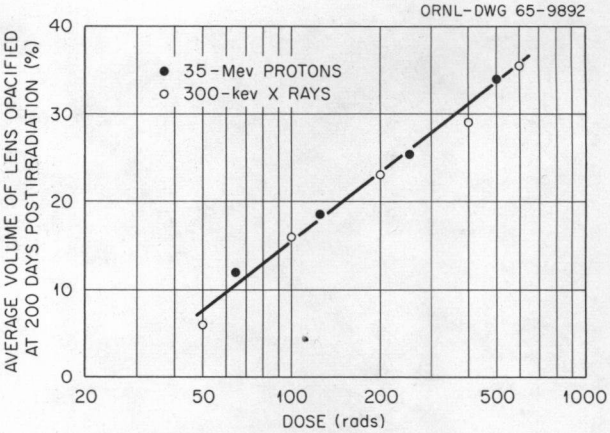

Fig. 11.2.3. Average Severity of Lens Opacities at 200 Days After Irradiation in Relation to the Dose Produced by Either Protons or X Rays.

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11.3 GENETIC EFFECTS OF SIMULATED FLIGHT CONDITIONS ON THE PROTOTYPE PACKAGES FOR THE *NEUROSPORA* BIOSATELLITE EXPERIMENT

F. J. de Serres B. B. Webber

In preparation for the biosatellite experiment in June 1966, ground-based experiments are being performed both to test the design of the *Neurospora* prototype packages and to provide a preliminary biological assay of the effects of the acceleration, impact, and vibrational parameters, alone and in combination with radiation. In April 1965 a testing program was designed to provide such an assay. The genetic effects on *Neurospora* conidia in prototype packages subjected to (1) simulated flight profile alone, (2) radiation profile alone, or (3) the simulated flight profile in combination with the radiation profile were compared with (4) untreated controls.

Neurospora conidia were packaged and transported in the prototype packages. Treatments were designed to approximate the intensities of

vibration and the gravitational forces during acceleration and impact actually experienced during satellite launching and reentry. Gamma-ray doses from an ^{85}Sr source were administered continuously over a period of about 66 hr. These were estimated to be 2900, 1670, and 640 rads for three groups of conidia subjected to radiation alone, and 2270, 1400, and 555 rads for three groups of conidia subjected to radiation and the simulated flight profile. The frequency of forward mutation in the *ad-3* region among the heterokaryotic surviving conidia (as described previously)[1] was used to assay the genetic effects of the four different treatment conditions.

Results and Conclusions. – In Fig. 11.3.1 the mutation frequencies are plotted against estimated doses for the irradiated conidia. Unirradiated conidia (with or without vibration and centrifugation) typically exhibited forward-mutation frequencies of about 0.5 mutant per 10^6 survivors. There is no evidence for an enhancement of the mutagenic effect of gamma radiation by the vibration and centrifugation profiles used. Included in Fig. 11.3.1 are two estimates of mutation rate from a low-intensity 250-kvp x-ray experiment carried out under laboratory conditions, which indicate that the quantitative results from the gamma experiment are consistent with our expectations.

It may be concluded that the *Neurospora* conidia maintain approximately normal viability and radiosensitivity during storage in the *Neurospora* prototype packages for the period of time required for the proposed biosatellite experiment. Furthermore, the experimental results permit the tentative prediction that the mutagenic effectiveness of low-intensity gamma irradiation should not be affected by the vibration and forces of acceleration associated with launching and reentry of the biosatellite.

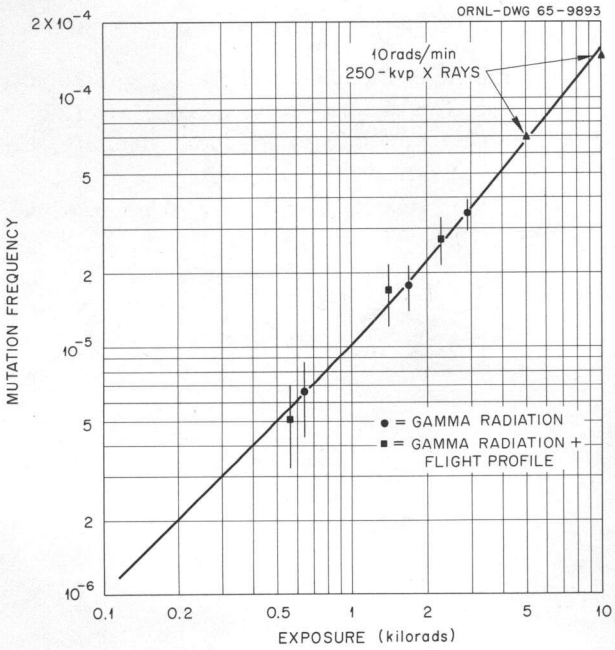

Fig. 11.3.1. Forward-Mutation Frequencies Obtained with the *Neurospora* Test System with Gamma Radiation Alone or with Radiation in Combination with Simulated Flight Profile as Compared with Forward-Mutation Frequencies from Exposures to 250-kvp X Rays (10 r/min).

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11.4 GENETIC EFFECTS OF A SIMULATED FLIGHT PROFILE ON PROTOTYPE PACKAGES FOR THE *HABROBRACON* BIOSATELLITE EXPERIMENT

R. C. von Borstel
Anna R. Whiting
Sohei Kondo[1]
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Mary Lou Pardue[2]

Introduction. – The biosatellite experiment is designed to test synergistic effects of weightlessness and radiation, as well as other possible spaceflight conditions, in a three-day satellite that will contain its own radiation source as one of the controls. A number of organisms, including the parasitic wasp *Habrobracon*, are being prepared for the experiment expected to be held in June 1966.

The purpose of this ground-based experiment was to test the radiation subassembly and prototype packages with *Habrobracon* in conjunction with all other radiation experiments under four separate conditions. These conditions were (1) the simulated flight profile (centrifugation and vibration), (2) the gamma irradiation (from a low-level ^{85}Sr gamma source), (3) the simulated flight profile plus the irradiation, and (4) the nonprofile-nonirradiation control. It was assumed that this would provide an

adequate test of the flight hardware, as well as provide preliminary ground control data for the eventual orbital flight.

Dosimetry. – Toshiba glass rods were used to measure the doses. Their readings were standardized by glass rods exposed to an NBS ^{60}Co gamma field. Their energy dependence was checked by an ORNL ^{137}Cs gamma field calibrated by a Victoreen chamber which, in turn, was standardized by NBS. There was no energy dependence. These rods were either encased in nylon, in polyethylene tubes, or in aluminum. Other glass rods encased in plastic, lead, or copper were used to make qualitative estimates of the amount of scattered radiation. Dosimetry was performed only on the packages receiving the gamma radiation, not on the control packages or on the profile-only packages.

It was found that the radiation subassembly does not follow dose-square relations. From the ratios of the measurements of dosimeters encased in plastic, lead, and copper, it seems that the scattered gamma radiation does not contribute too much to the total dose (<10%). Precise determination of the contribution of scattered radiation of different wavelengths would require extensive further experimentation.

Results. – From the data obtained and by using the equations of von Borstel and Rekemeyer,[3] a dose-action curve for sperm survival (1.0 – dominant lethality) was constructed. This is shown in Fig. 11.4.1. It is apparent that there is no clear evidence for a synergistic effect for the flight profile and the radiation for induced dominant lethality in sperm.

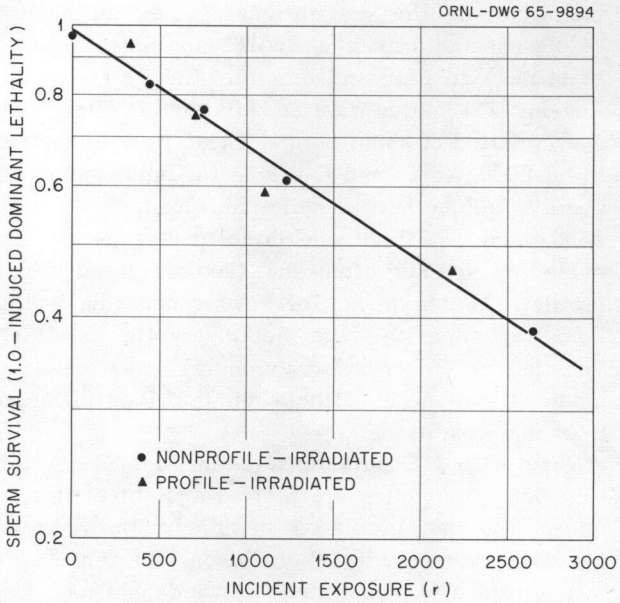

Fig. 11.4.1. Dose Response for Embryo Dominant Lethality Induced in Sperm.

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Cell Physiology

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12.1 DENSITY-GRADIENT PROFILES OF OSMOTIC PHENOMENA IN BACTERIOPHAGES

S. P. Leibo Peter Mazur

It has been demonstrated previously that bacteriophages T4B and $T4BO_1$ differ with respect to their survivals when exposed to an "osmotic shock" treatment.[1] In the case of T4B, survival is primarily dependent on the osmotic pressure differential between the internal environment of the phage head and the suspending medium, and secondarily on the final salt concentration of the suspending medium following dilution. More recent experiments show that the osmotic pressure differential and not the dilution volume is the critical factor in osmotic shock of phage T4B. In the case of $T4BO_1$, in which the osmotic effect is not operative, survival is wholly dependent on the final salt concentration of the medium following dilution. Density-gradient separations have now been performed to determine if a loss of survival of $T4BO_1$ is correlated with a loss of DNA from the phage head.

Phage T4B was suspended at a concentration of 2×10^{13} particles/ml in 3.0 *m* NaCl and was diluted either slowly or rapidly to 0.003 *m* NaCl. These dilution rates result in survivals of 100 and 1%, respectively, for T4B.[1] Phage $T4BO_1$ at a concentration of 2×10^{13} particles/ml in 3.0 *m* NaCl was diluted rapidly to 0.3 and 0.0003 *m* NaCl. These final salt concentrations result in survivals of $T4BO_1$ of 95 and 10% respectively.[1] The diluted phages were layered over CsCl and centrifuged in a Spinco SW 39L rotor at an average force of $85,000 \times g$ for 20 hr at 15°C. Fractions were monitored and collected according to the technique of Fisher *et al.*[2] The results are shown in Fig. 12.1.1*a* and *b*. Control samples of the two phages suspended in and diluted with 0.1 *M* phosphate buffer, pH 7.0, plus 0.01 *M* Mg^{2+} were also centrifuged separately and as a mixed population in a CsCl gradient. The profile of the mixed population is shown in Fig. 12.1.1c. These data demonstrate the following points:

1. A portion of the population of T4B loses DNA when subjected to osmotic shock, whereas $T4BO_1$ loses little or no DNA when exposed to the same treatment. This significant point substantiates the previously suggested view[1] that the loss of survival of $T4BO_1$ which accompanies osmotic pressure change is not "osmotic shock."
2. The density of phage $T4BO_1$ previously suspended in phosphate buffer is greater than that of phage T4B suspended in buffer. (The identities of the two peaks in Fig. 12.1.1c were determined from the density profile when each phage was centrifuged separately.)
3. The density of T4B previously suspended in 3.0 *m* NaCl is greater than the density of T4B previously suspended in 0.1 *M* phosphate buffer.
4. The density of $T4BO_1$ previously suspended in 3.0 *m* NaCl is also greater than that of $T4BO_1$ previously suspended in phosphate buffer.

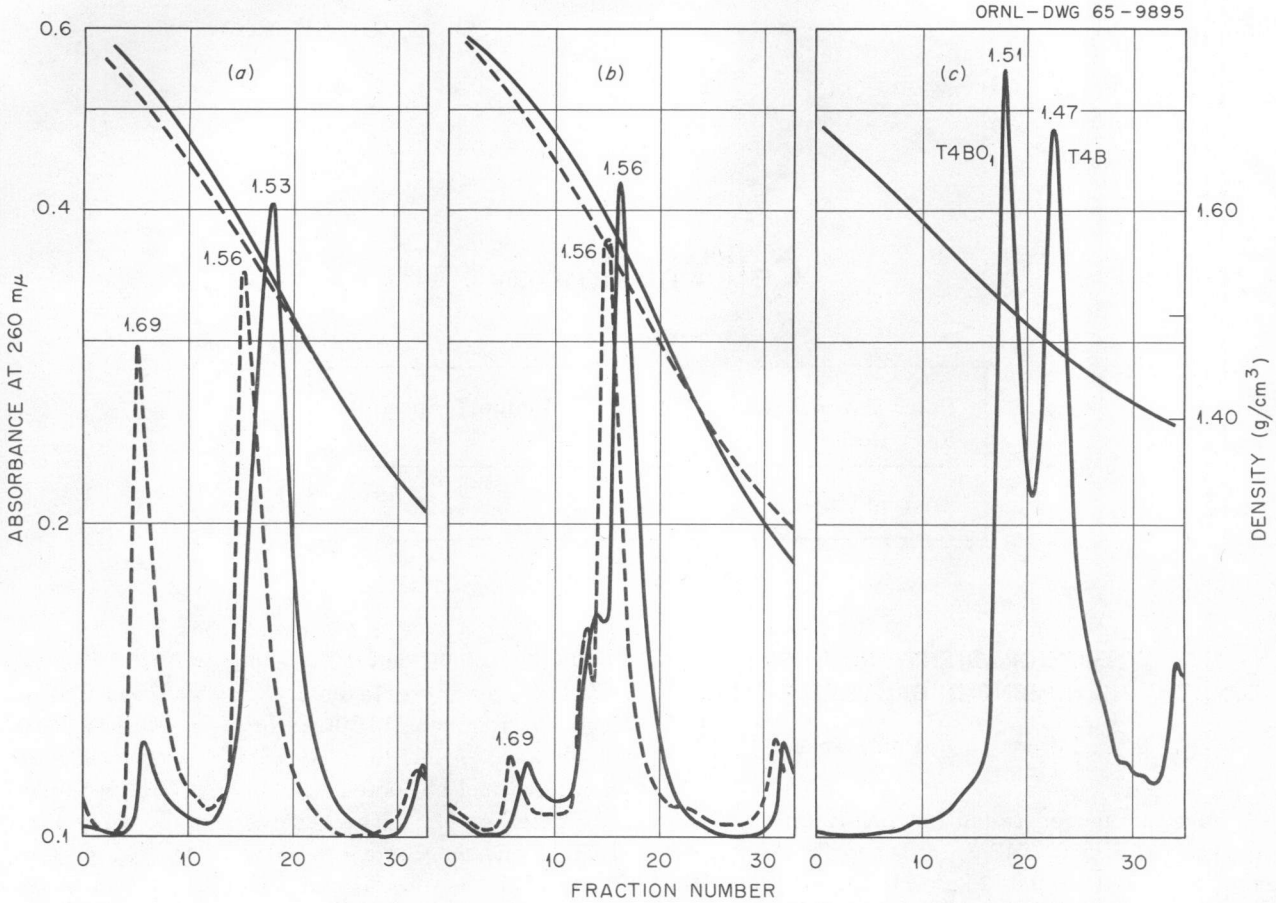

Fig. 12.1.1. Cesium Chloride Density Gradient Profiles of Bacteriophages T4B and $T4BO_1$. (*a*) Solid line: T4B following slow dilution from 3.0 to 0.003 *m* NaCl; broken line: T4B following rapid dilution from 3.0 to 0.003 *m* NaCl. (*b*) Solid line: $T4BO_1$ following rapid dilution from 3.0 to 0.3 *m* NaCl; broken line: $T4BO_1$ following rapid dilution from 3.0 to 0.0003 *m* NaCl. (*c*) T4B and $T4BO_1$ suspended in 0.1 *M* phosphate buffer, pH 7.0. The numeral above each peak represents the average density of that component in three experiments. The solid- and broken-line density profiles correspond to the solid- and broken-line absorbance profiles.

5. The density of $T4BO_1$ previously suspended in 3.0 *m* NaCl is greater than that of T4B previously suspended in the same solution.

6. The density of osmotically shocked but still intact T4B is greater than the density of unshocked T4B exposed to the same initial and final salt concentrations.

The density differences described above can be explained adequately in terms of the well-documented difference in permeability between T4B and $T4BO_1$.[1,3] For example, since $T4BO_1$ is more permeable than T4B, when centrifuged in CsCl, $T4BO_1$ will probably contain more Cs^+ than T4B does and will, therefore, be more dense. Further, since the membrane of T4B is undoubtedly damaged by osmotic shock, Cs^+ probably replaces less-dense cations when shocked T4B is centrifuged in CsCl. One unexplained density difference, however, is that which is found for both T4B and $T4BO_1$ when these phages have been exposed to concentrated NaCl prior to centrifugation in CsCl. Nevertheless, all these density differences add further support to the contention that there is solvent water present within the head of T-even phage.

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12.2 QUANTUM YIELD FOR THE ULTRAVIOLET-INDUCED DIMERIZATION OF THYMINE IN FROZEN SOLUTION

Peter Mazur Walter Füchtbauer[1]

A preliminary estimate of the quantum yield for the dimerization of thymine in frozen solution was given in the previous semiannual report;[2] that estimate has now been recalculated on the basis of additional experiments and a correction for the scattering by ice.

The quantum yield, the number of affected molecules per absorbed quantum, is

$$\phi = \frac{dc}{dt}\,\frac{V}{A(I_{\text{abs}})}\,, \tag{1}$$

where c is the concentration of thymine (moles/cm^3), t is the duration of irradiation (sec), V is the volume of irradiated material (cm^3), A is the irradiated area (cm^2), and I_{abs} is the rate at which the incident light is absorbed per unit area of frozen solution (einsteins cm^{-2} sec^{-1}).

But I_{abs} can be expressed in terms of the intensity of the incident radiation (I_0), the emergent radiation picked up by the detector (I_D), and the radiation that is lost (i.e., neither absorbed nor detected), I_L; namely,

$$I_{\text{abs}} = I_0 - I_D - I_L\,. \tag{2}$$

Scattering by the ice crystals is responsible for I_L; however, not all the scattered radiation is necessarily lost; some of it may be absorbed by the thymine. This possibility can be expressed as

$$I_L = aI_s\,, \tag{3}$$

where I_s is the intensity of the scattered radiation, and a is that fraction of I_s neither detected nor absorbed and has a value between 0 and 1.

Both I_D and I_s were actually measured in terms of transmittances. Thus,

$$I_D = I_0 T_{FT}\,, \tag{4}$$

where T_{FT} is the measured transmittance of the frozen thymine solution. And

$$I_s = I_0(1 - T_{\text{ice}})\,, \tag{5}$$

where T_{ice} is the transmittance of a sample of pure water frozen under the same conditions as the thymine solution.

If Eqs. (2), (3), (4), and (5) are substituted into Eq. (1), we obtain

$$\phi = \frac{dc}{dt}\,\frac{X}{I_0(1 - T_{FT}) - aI_0(1 - T_{\text{ice}})}\,, \tag{6}$$

where X is the sample thickness ($= V/A$).

Table 12.2.1 lists the values of the various variables in Eq. (6) for three experiments, and Table 12.2.2 gives the calculated quantum yields. As can be seen, ϕ is sensitive to the value of a assumed. If it is assumed that all the scattered light was absorbed by thymine ($a = 0$), ϕ is between 0.73 and 1.11 molecules of thymine dimerized per quantum absorbed. If it is assumed that all the scattered light was lost and none absorbed ($a = 1$), ϕ is between 2.7 and 4.6.

It is difficult to believe that a can be zero, that is, that all the radiation scattered by ice was absorbed during its passage through the frozen solution of thymine.

It is equally difficult to believe that a can be 1, that is, that all the radiation scattered by ice was lost and none absorbed by thymine. Some scattering must occur in the deeper layers of the sample, and some of the scattered photons almost certainly would have to hit thymine clumps while traversing the sample. Furthermore, an assumption that $a = 1$ leads to quantum yields of 3 to 4, which would mean that a photon absorbed by one thymine molecule produces $1\frac{1}{2}$ to 2 dimers. This possibility sounds unreasonable.

If we assume that the quantum yield is 2, the values of a fall between 0.64 and 0.84; that is, between 64 and 84% of the scattered radiation is lost. In the absence of a mathematical analysis of the scattering or an experimental determination of it, all we can say is that these latter values appear reasonable. Hence, our guess is that the

Table 12.2.1. Values of Parameters Used in Calculating Quantum Yields for Dimerization of Thymine in Frozen Solution

Experiment	Irradiation Temperature (°C)	I_0 (einsteins cm^{-2} sec^{-1})	X (cm)	dc/dt (moles cm^{-3} sec^{-1})	T_{FrzThy} [a]	T_{ice} [a]
		$\times 10^{-10}$		$\times 10^{-9}$		
I	−5	2.60	0.115	1.92	0.236	0.550
II	−70	2.60	0.115	1.92	0.153	0.337
III	−10	3.49	0.115	1.40	0.369	0.522

[a]Relative to liquid water.

Table 12.2.2. Estimated Quantum Yield for Dimerization of Thymine in Frozen Solution

Experiment	Irradiation Temperature (°C)	Value of ϕ when		Value of a when	
		$a = 0$	$a = 1$	$\phi = 1.11$	$\phi = 2.00$
I	−5	1.11	2.70	0	0.75
II	−70	1.00	4.62	0.12	0.64
III	−10	0.73	3.01	0.45	0.84

true quantum yield for dimerization lies closer to 2 than to 1. A value of 2 would mean that one dimer is formed for each photon absorbed by a thymine molecule. Or to put it differently, it would mean that only one thymine molecule need be excited to form a dimer, and if it is excited, a dimer will form.

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[1]Present address: Botanisches Institut der Universität, Würzburg, Germany; work done while at ORNL.

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12.3 EFFECT OF COOLING AND WARMING VELOCITY ON THE SURVIVAL OF FROZEN AND THAWED YEAST

Peter Mazur Janice J. Schmidt

Figure 12.3.1 summarizes the results obtained to date on the relation between the survival of

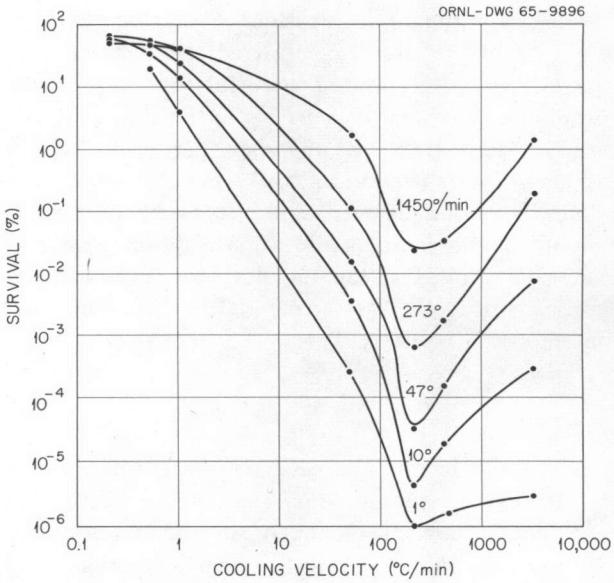

Fig. 12.3.1. Survival of Yeast as a Function of Cooling Velocity to −70°C. Cells were cooled at indicated rates to −70°C, then cooled rapidly to −196°C for 10 min, and warmed rapidly to −70°C. The numbers on the curves refer to the rate of warming from −70 to 0°C.

yeast and the rate at which it is cooled to and rewarmed from −70°C. It will be noted that lethality and the relative effect of warming velocity both increase as the cooling velocity is increased from 0.2 to 250°C/min. Small variations in cooling velocity around 50°C/min apparently affect survival greatly. Five replicate experiments using that nominal rate yielded survivals that varied considerably in absolute value, although all the experiments agreed in that survivals increased progressively with increasing warming velocity. Since the drop in survival has been shown to be associated primarily with intracellular freezing,[1,2] it appears that the range between 1 and 50°C/min is particularly critical in determining whether lethal quantities of intracellular ice form. Procedures have been developed to investigate the effect of cooling at rates between 1 and 50°C/min.

Survival is apparently minimal at a cooling velocity of 200°C/min and rises at higher cooling velocities. The highest cooling velocities indicated in Fig. 12.3.1 were obtained using the centrifuge spray technique described previously.[3] Since we have not been able to measure the numerical value of the cooling velocity achieved with this technique, we do not know the true shape of the right-hand ascending limbs of the curves. Therefore, procedures are being developed to cool samples at measurable rates between 500 and 5000°C/min to explore this region of the curves more precisely.

As indicated previously,[1,2] our current explanation for the increasing survivals at very high cooling velocities is that these cooling velocities produce intracellular ice crystals, most of which are sufficiently small to be innocuous. Because they are small, they can grow to damaging size if warming is carried out sufficiently slowly, but may not grow to damaging size if the warming is carried out rapidly.

As shown in Fig. 12.3.2, the effects of cooling and warming velocity depend also on the temperature to which the cells are cooled. Between 0 and −15°C, survival drops from 100 to about 50%, regardless of the cooling and warming velocity. The survival of rapidly cooled cells continues to drop precipitously with a further lowering of temperature and falls below 10% at −20°C. We have interpreted this latter drop as being due to intracellular freezing. Slowly cooled cells behave differently. Those cooled slowly and warmed slowly show a second drop in survival from 50 to 10%

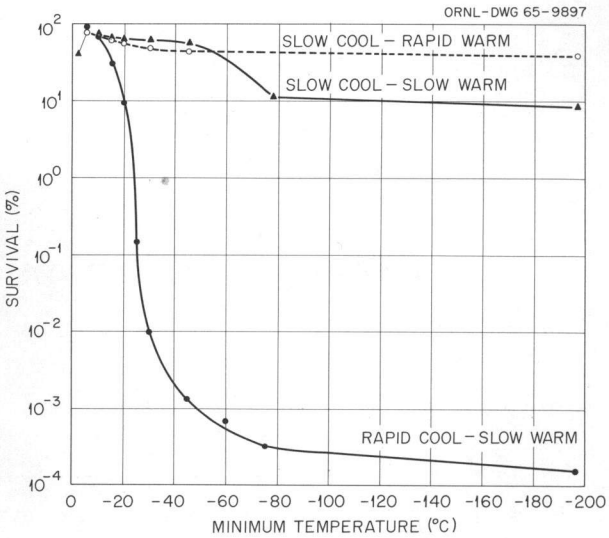

Fig. 12.3.2. Survival of Yeast as a Function of the Minimum Temperature to Which the Cells Were Cooled. Rapid cooling was 20 to 50°C/min. Slow cooling was 1.4°C/min. Slow warming was 1.0°C/min, and rapid warming was 500 to 1400°C/min.

between −45 and −78°C, some 25° below the temperatures producing the abrupt drop with rapidly cooled cells. This second drop, however, does not occur if the slowly cooled cells are subsequently warmed rapidly instead of slowly. Our tentative hypothesis is that this second drop is due either to the eutectic crystallization of the residual free water in the cells or to the crystallization of some of the bound water. Calorimetric measurements of the amounts of ice forming in cells as a function of temperature should permit testing these possibilities. The construction of the calorimeter is now complete, and it is shortly to be calibrated.

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Chemical Protection and Enzyme Catalysis

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13.1 CHEMICAL PROTECTION

D. G. Doherty Margaret A. Turner
Vu-Thi-Suu

We have been examining the effects of multiple chemical treatments on the degree of radiation protection obtained at high radiation dose levels in an effort to determine both the relative contribution of each chemical and the organ system protected. As a measure of protection to the hematopoietic system, the addition of isogenic bone marrow after irradiation should show little additive effect if the main chemical protection is exerted in the marrow. This question raised the issue of the degree of recovery that can be obtained with marrow alone, as well as the most effective or optimal cell dose for maximum recovery. This was explored by determining the radiation $LD_{50/30}$ for BC3F1 mice, using various bone marrow cell doses injected intravenously into groups of approximately 100 animals 24 hr after x irradiation. One to two male and female runs were made at each cell dose. The $LD_{50/30}$'s were obtained from a probit analysis of the results made on a Control Data Corporation 1604 computer.

No sex difference in recovery was observed at any of the cell doses, so the data were pooled; the calculated values are shown in Fig. 13.1.1. Interestingly, the response obtained is similar to that seen for drug action in that it is proportional to the logarithm of the cell dose administered. The biphasic nature of the curve is indicative of two separate responses to marrow transfusion – one similar to the radiation sensitivity of the marrow and the other to the sensitivity of the intestine. The first may be interpreted as the rapid and easy restoration of the marrow by relatively small cell doses of the order of 100,000 to 500,000 cells. The second response would seem to be the abscopal effects of a viable marrow on the recovery of the intestine and/or the additional ability of large marrow transfusions to contribute directly to

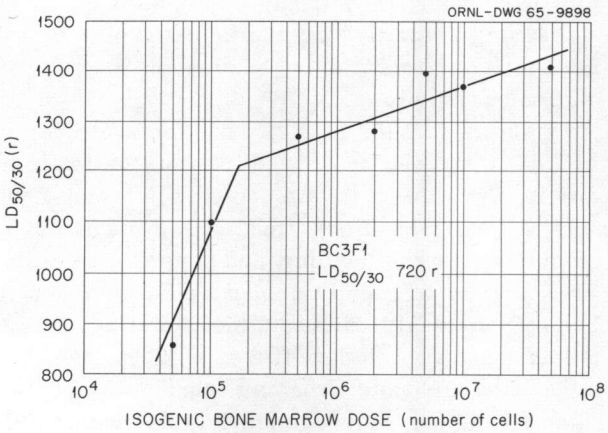

Fig. 13.1.1. Radiation $LD_{50/30}$ of BC3F1 Mice Treated with Isogenic Bone Marrow Suspensions.

intestinal recovery. Increasing the cell dose by factors of 10 to 100 only served to increase the $LD_{50/30}$ by, at most, 150 r. At high radiation doses, intestinal recovery seems to be a limiting factor. Thus the optimal marrow cell dose for maximum recovery in these mice is 5×10^6 cells. All the animals have been kept for 90 days in order to observe any delayed effects, and calculations of an $LD_{50/90}$ are in progress.

13.2 CHROMATOGRAPHIC STUDIES OF MOUSE KIDNEY MURAMIDASE

Vu-Thi-Suu

Introduction. – It was reported earlier[1] that an analytical study of normal mouse kidney muramidase, using chromatographic techniques, was being investigated.

We have concentrated our attention on the improvement of methods to minimize the loss of enzyme in the extraction, desalting, and the lyophilization techniques.

Methods and Results. – Both kidneys from 1125 mice 1C3F1/Cum, male and female, 3–12 months old, were excised, wrapped in aluminum foil, and frozen in dry ice until assayed. A modification of the method of Jollès[2] was used in this study. Muramidase activity was determined photometrically according to the method of Shugar.[3] Protein content was determined by the Folin-Ciocalteu reagent[4] or extinction at 280 mμ. Kidneys were thawed at room temperature, then homogenized with phosphate buffer *M*/15, pH 7.3, using a sonifier, and centrifuged at high speed. The supernatant, brought to pH 4.5, was heated 75 sec in a boiling water bath, chilled rapidly, and centrifuged. The sediment was discarded. The supernatant was stirred overnight with Amberlite CG-50 resin buffered with 0.2 *M* phosphate buffer, pH 6.5. The resin was washed with water, and the enzyme eluted with 0.6 *M* phosphate buffer, pH 6.5. The eluate was then desalted by filtration through Sephadex G-25. Most of the salt was removed, and the recovery of the muramidase activity was 70–80%. The desalted muramidase is, at present, being chromatographed on carboxymethylcellulose resin columns.

Discussion. – As reported earlier,[1] because of the very low concentration of muramidase in normal mouse kidney, the main technical problem in this study is to minimize the loss of the enzyme during the different steps of extraction, desalting, and lyophilization.

By extracting with M/15 phosphate buffer, pH 7.3, instead of water as Jollès used in his method,[2] a five- to sixfold increase in muramidase activity has been obtained.[1] The desalting by dialysis, using cellophane or collodion bags, has led to a loss of 50–90% of muramidase activity, and many attempts to lyophilize the extract have led to a loss of 50% of the enzyme activity. By using the filtration through Sephadex G-25, a much higher yield in enzyme activity has been obtained. This filtration has been made with a 90 cm × 7 cm^2 column, thus making unnecessary the lyophilization, and consequently avoiding any loss of enzyme activity.

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Mammalian Recovery

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Secondary Disease (14.1–14.3)

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D. C. Swartzendruber[a]
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Recovery in vitro (14.4)

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Hemopoietic and Lymphatic Stem Cells (14.5–14.7)

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Animal Testing Program (14.9)

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14.1 LYMPH NODE CHANGES IN RADIATION CHIMERAS

C. C Congdon Kathleen H. Harrington

Introduction. – Most of our recent studies on secondary disease in lethally irradiated mice treated with foreign bone marrow have dealt with metabolic aspects of the disease and with 90-day mortality. Earlier investigations were concerned with the pathologic anatomy of secondary disease.[1] In the present work a new morphologic study has been initiated on the lymphatic tissue changes in isologous and homologous mouse radiation chimeras. These tissues were chosen for special investigation because the earlier studies indicated that the major site of graft-vs-host reactions is lymphatic tissue.

Materials and Methods. – Enough 950-r x-irradiated $1C3F_1$ female mice were given 10 million $B6D2F_1$ bone marrow cells (HBM) intravenously so that five mice could be autopsied

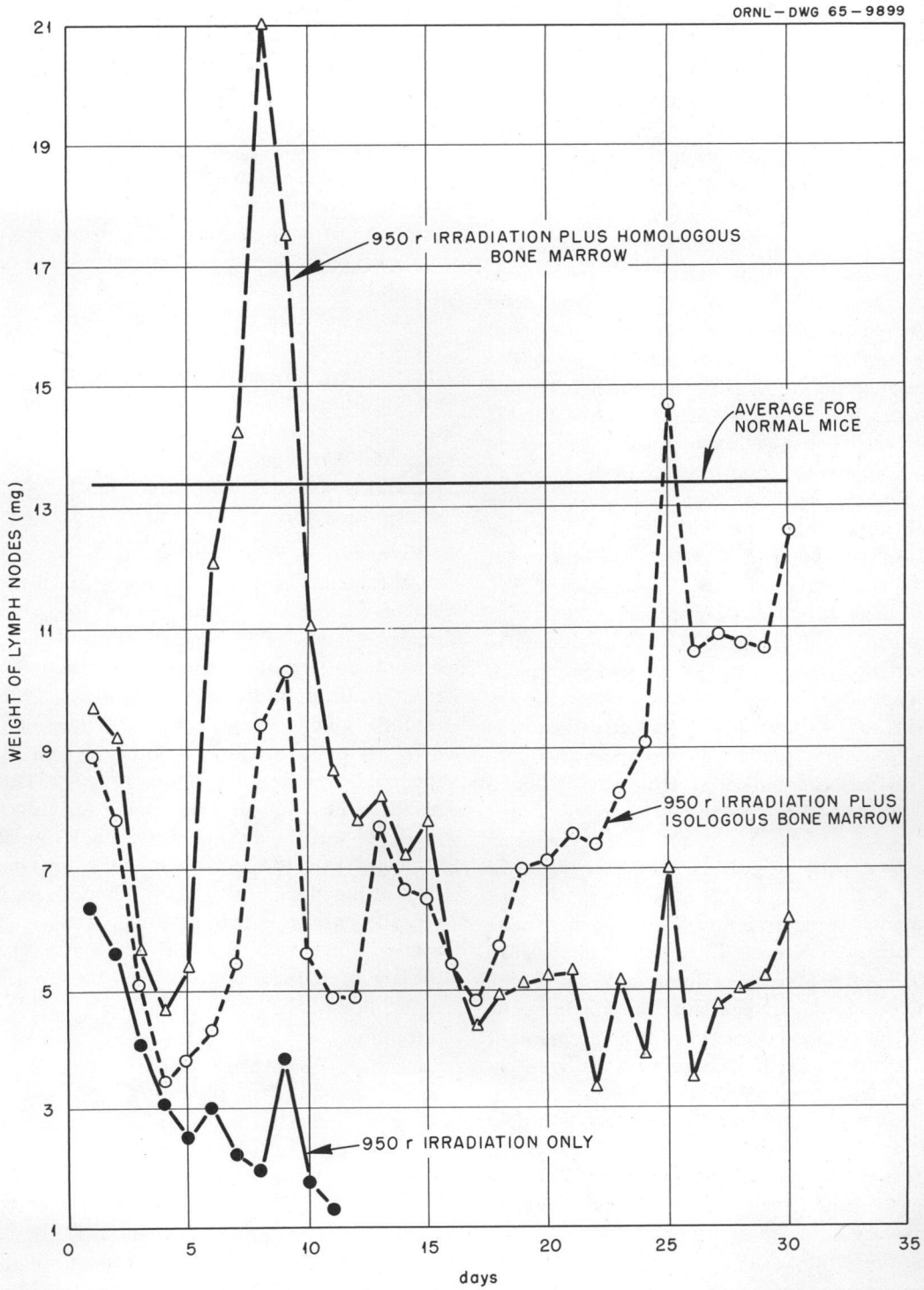

Fig. 14.1.1. Weight Changes in Four Peripheral Lymph Nodes in Mice Exposed to Whole-Body X Rays, Then Given 5×10^7 Homologous Bone Marrow Cells Intravenously. The weight increase 5 to 10 days after exposure represents the proliferative phase of the graft-vs-host reaction. Extreme atrophy follows this phase. Also shown are the weight changes in lymph nodes in mice similarly irradiated but given isologous bone marrow afterwards. The temporary weight increase between 5 and 10 days after treatment is caused by extramedullary granulopoiesis and does not represent a graft-vs-host proliferation. Each point usually represents five mice.

each day for 30 days. Pooled weights of the two brachial and two inguinal lymph nodes were obtained, and the nodes were fixed for histologic study. Similar data were obtained on normal mice, x-ray controls, and those given isologous bone marrow (IBM). Additional experiments of the same design were carried out with donor bone marrow cell doses of 50 million and 1 million cells.

Results. – In the x-ray controls, lymph node weight decreased. In IBM mice, lymph node weight decreased and then returned to normal values during the fourth week after x ray. The HBM mice showed a marked increase in lymph node weight by 10 days after treatment, and extreme atrophy had developed at 30 days. Some HBM mice, however, had normal weight at 30 days, and there was a great deal of scatter throughout the data. The early weight increase at 10 days was correlated with proliferation of pyronin-staining cells in the tissue, while the extreme atrophy at 30 days was associated with loss of normal architecture. The HBM mice without atrophic nodes near the 30-day period showed normal regenerating lymph nodes. These animals are thought to be examples of primary induction of tolerance in adult mice and support the concept of two major populations (tolerant and nontolerant) within the HBM group.

Figure 14.1.1 shows the lymph node weights in the experiment with 50 million donor bone marrow cells.

Discussion. – The findings on lymph node weight changes in three different bone marrow cell dose experiments are of interest because the greatest (5- to 10-day) lymph node weight increase in homologous chimeras occurred with the greatest cell dose. Presumably, this represents the greatest graft-vs-host reaction. Other studies, however, have demonstrated that death from graft-vs-host reactions does not depend on bone marrow cell dose.[2] This discrepancy in the outcome of lymph node changes in relation to cell dose and mortality from secondary disease in relation to cell dose requires further study.

References

[1]C. C Congdon and Irene S. Urso, *Am. J. Pathol.* **33**, 749–67 (1957).

[2]C. C Congdon, M. A. Kastenbaum, and D. A. Gardiner, *J. Natl. Cancer Inst.* **35**, 227–34 (1965).

14.2 RESPONSE OF RADIATION CHIMERAS TO REPEATED BLEEDING

A. L. Kretchmar[1]

Introduction. – In studying a model of the erythropoietic system from the point of view of mammalian recovery after total-body irradiation and the injection of bone marrow cells, it became apparent that a normal peripheral blood picture would be possible even though complete recovery in the immature ("stem cell") cellular pools had not taken place.[2] It was only necessary to change the relative probabilities of differentiation from, and proliferation in, the stem cell pool in order to obtain complete peripheral recovery with an incompletely regenerated marrow population. This possibility was investigated in the experiments summarized here.

Methods. – Radiation chimeras were produced in the usual way by injecting 20 to 40 $\times 10^6$ homologous bone marrow cells into lethally irradiated mice. The bone marrow cell donors were normal unirradiated mice (isologous or homologous). Irradiated mice given similar numbers of isologous bone marrow cells and normal untreated mice were controls. All animals were bled from the ocular sinus in a standard way. Hematocrit, hemoglobin, serum iron, and reticulocyte percentage were determined on each animal using standard hematologic techniques. One-half milliliter of blood was drawn into a heparinized Pasteur pipet from each animal at each bleeding. Bleeding was at three- and four-day intervals (alternating) for five bleedings, beginning on the days indicated in Table 14.2.1.

Results. – Table 14.2.1 presents a summary of the data. Removal of blood at this rate (approximately one-third of the total blood volume twice a week) constitutes a strong stimulus to erythrocytic differentiation, and normal mice can maintain a daily production of new red cells that keeps a reticulocyte volume of 7 to 11% of the whole blood volume. Despite this elevated production of red cells, fully normal hemoglobin and hematocrit levels cannot be maintained, and the values fall to 13–14.5 g per 100 ml and 45–48%, respectively, from 16–17 and 48–52, which are the prebleeding levels in normal mice. The schedule, therefore, constitutes a maximal stimulus to erythropoiesis without unduly depressing peripheral RBC levels.

Table 14.2.1. Response of Radiation Chimeras to Bleeding[a]

Factor	Group	Time After X Ray and Bone Marrow (days)						
		7	22	37	76	132	176	212
Hematocrit	Normal	46	45	45	47	48		45
	IBM	44	44	41	44	40		
	HBM	38	24	21	37		34	40
Hemoglobin	Normal	14	13.5	13	14.5	13.5		13
	IBM	13	12.5	12.5	13.5	11.5		
	HBM	11	7.5	6	11		10	11.3
Reticulocyte Response[b]	Normal	6.7	9	7	9.5	11		11
	IBM	8.5	5.5	6	9.2	10.2		
	HBM	6.7	2.5	2.0	7.5		7.1	11

[a]The data are the average of the values determined at the 3d and 4th bleeding, when the response was more or less stabilized. The prebleeding values were: normal, 48–52, 16–17, 0.5–1.0; IBM, 48–54, 16–17, 1–2; and HBM, 46–54, 13–17, 1–2.

[b]The value shown is an indication of the absolute reticulocyte response and is obtained by multiplying the hematocrit by the fraction of the circulating red blood cells that are reticulocytes, as determined from a cresyl-blue-stained blood smear.

In irradiated mice given isologous bone marrow, the hematocrit and hemoglobin levels before bleeding were within normal limits at all times tested (7 to 132 days after irradiation and bone marrow). The response to bleeding was good but slightly subnormal. It is essentially interesting to note that regeneration of the marrow plateaus (20 to 40 $\times$ 10^6 cells injected on the day of irradiation) by day 7 and does not show significant improvement even as long as 132 days after irradiation. Thus the marrow does not fully recover when a dose of 20 to 40 $\times$ 10^6 isologous bone marrow cells is used.

When the same number of homologous cells are given, the response to bleeding is not so good as in the isologous-marrow-treated groups. There is rapid initial recovery of the marrow, with a secondary loss in erythropoietic capacity in the 20- to 40-day interval, and not until 212 days after irradiation and bone marrow treatment does the erythropoietic response of homologous chimeras equal that of mice treated with isologous bone marrow cells.

Since one-third of the blood volume is removed at the first and each subsequent bleeding, the fact that host RBC are still circulating at seven days after irradiation does not interfere with the evaluation of erythropoietic response capacity, because any new red cell formation must come from the proliferating graft. The progressive decline in values or plateau at low levels that occurs in the chimeras at the third, fourth, and fifth bleedings even with maximal (though subnormal) reticulocyte response indicates that erythropoietic capacity is less than that of isologous-marrow-treated mice and is severely reduced at 22 and 37 days.

Serum iron levels were reduced in all groups to 1–2 gammas/ml from the prebleeding levels of 2–3 gammas/ml. Supplementary iron feeding by addition of ferrous sulfate to the drinking water did not alter the results. In general, levels were higher in chimeras than in normal mice at the third and fourth bleedings, with the levels for irradiated mice given isologous cells being intermediate. Thus the serum iron levels were lowest in normal mice, where erythropoietic capacity was greatest, and highest in the chimeras, where erythropoietic capacity was reduced.

Conclusion. – Irradiated mice given isologous or homologous bone marrow cells do not have completely regenerated marrow cell populations even though they may show complete recovery to normal values in hematocrit, hemoglobin, reticulocyte, and serum iron determinations. Approximately 200 days are required for chimeras to

achieve a bone marrow cell population with as great an erythropoietic capacity as irradiated mice given isologous cells. Recovery in mice given isologous cells is rapid but not complete even at 132 days after irradiation. The erythropoietic capacity of chimeras shows a marked secondary reduction at the 20- to 40-day interval.

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14.3 PARASITES IN THE ALIMENTARY TRACT OF RADIATION CHIMERAS

W. H. McArthur[1]

Introduction. – It is paradoxical that irradiated mice given HBM maintain positive nitrogen balance, yet lose weight.[2] We suggested that this occurs because nitrogen is retained in some abnormal compartments. Since these compartments may include parasites and bacteria of the alimentary tract, studies were made to determine quantitatively the parasitic fauna of radiation chimeras.

Materials and Methods. – A total of 61 $1C_3F_1$/Cum animals (male and female) 14 to 15 weeks of age were used in these experiments. Homologous animals received 950 r of x irradiation and $B_6D_2F_1$/Cum bone marrow cells (20×10^6). Animals were sacrificed by decapitation on days 7, 14, 22, 45, and 70. The alimentary tracts were removed and slit open. The contents of the tracts from the esophagus through the large intestines less the cecum were placed in 0.9% saline and examined under the dissecting microscope. Smears of the cecal content were made and immediately examined for parasites. Some of the smears were fixed in either Schaudinn's or Hollande's fixative and stained in Mayer's haemalum or iron-hematoxylin for more positive parasite identification.

Results and Discussion. – Observation of living material indicates that isologous and normal mice

Table 14.3.1. Parasitic Fauna of Radiation Chimeras

Group	Day	Average Number of Animals	Cecum Flagellates	Entire Alimentary Tract (number of parasites)	
				Nematodes	Cestodes
Normal	7	2	++++	0	0
Isologous		2	+++	0	0
X-Ray		2	++	75	3
Homologous		4	+	30	1
Normal	14	6	+++	0	0
Isologous		5	+++	46	0
Homologous		5	+++	13	0
Normal	22	3	++++	1	5
Isologous		3	+++	8	11
Homologous		4	None	74	14
Normal	45	4	++++	1	0
Isologous		5	++++	0	4
Homologous		1	++	0	27
Normal	70	5	+++	0	0
Isologous		5	+++	6	0
Homologous		2	+++	45	8

Note: ++++ represents caeca teeming with protozoan parasites; +++, half as many as found with ++++; ++, half as many as found with +++; +, a few scattered forms.

had more protozoan parasites than homologous mice through day 70 (Table 14.3.1). Protozoans in homologous mice appeared to be flagellates, including trichomonads, but a careful study of permanent slides is necessary for a more positive identification.

The data also show that normal mice were almost devoid of nematodes and cestodes. Natural resistance and acquired immunity normally keep the number of intestinal parasites under control. There is an increase in the number of helminths and nematodes in homologous mice. This finding parallels that of Friedberg *et al.*,[3] who reported that there is an increase in number of helminths and nematodes in mice with implanted rat marrow.

It is also noteworthy that there was a tremendous increase in the number of bacteria in both homologous and x-ray control mice.

References

[1]Knoxville College, Knoxville, Tennessee; work carried out in collaboration with Mammalian Recovery section.

[2]W. H. McArthur, C. C Congdon, and A. L. Kretchmar, *Exptl. Biol. Med.* **117**, 171 (1964).

[3]W. Friedberg, D. N. Faulkner, John K. Abbott, and Mary H. Friedberg, *Intern. J. Radiation Biol.* **8**, 101–10 (1964).

14.4 EFFECT OF TEMPERATURE ON X-IRRADIATED MOUSE BONE MARROW CELLS

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

Introduction. – We reported that erythropoietic activity of mouse bone marrow cells x irradiated and cultured in vitro was affected by post-x-ray culture temperature.[1] In terms of the potential of unirradiated cultured cells, irradiated marrow (100 or 150 r) retained more of its activity at 2 and 22°C than at 37°C. Additional results of the temperature effect have been obtained and are reported herein. The technique used to culture mouse marrow cells and to measure their erythropoietic activity was the same as previously described[2] except that 30-ml plastic tissue culture vessels were substituted for 25-ml Erlenmeyer flasks.

Results and Conclusions. – Figure 14.4.1 shows erythropoietic activity of cultured marrow cells

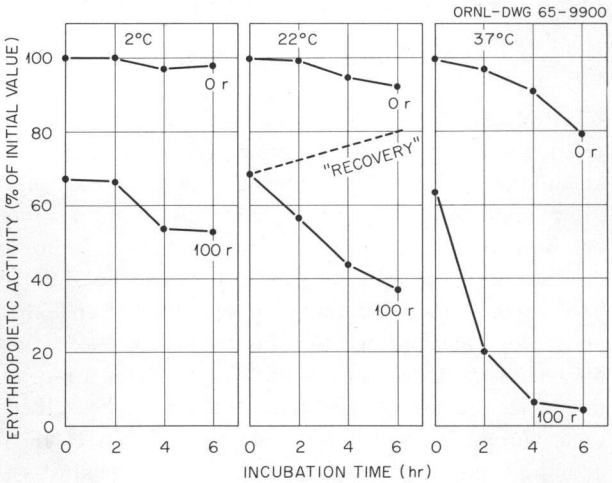

Fig. 14.4.1. Effect of Radiation on Marrow Cells as a Function of Culture Temperature.

as a function of incubation time at three temperatures. These results indicate that two factors contribute to the decline of activity. One involves inadequate culture conditions as evidenced by the decline of unirradiated cells, which is also temperature dependent. The second factor results from radiation injury, the degree of which is dependent on culture temperature. We interpret these results to mean that the sensitivity of irradiated erythropoietic stem cells to the adverse effects of culture is temperature dependent. Alternatively, these results may reflect the temperature dependence of development of the radiation damage.

We saw no evidence of repair of radiation damage, as indicated by the hypothetical dashed line in Fig. 14.4.1. It is possible that during the 6-hr incubation at 22 or 2°C, repair occurred but was overshadowed by factors attributable to inadequate culture conditions. Tests of culture temperatures between 22 and 37°C (2° intervals) also failed to reveal a type of temperature-dependent recovery which has been clearly demonstrated for bacteria and several other organisms.

References

[1]L. H. Smith and T. W. McKinley, Jr., *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1965*, ORNL-3768, p. 103.

[2]L. H. Smith and T. W. McKinley, Jr., *Biol. Div. Semiann. Progr. Rept. Aug. 15, 1963*, ORNL-3498, p. 102.

14.5 ERYTHROPOIESIS IN CHIMERIC MICE

Joan Wright Goodman Mysa C. Mathews

Introduction and Methods. – There are transplantation data which show that tumors and hemopoietic cells from certain parental strains grow less well in their F_1 hybrids than in mice of their own genotype. Because many of these studies are concerned primarily with the donor-host combination showing poor growth and the possible immunological interpretation of the phenomenon, it seemed advisable to look also at the performance of the other genotypes involved in a parent (P1)-parent-(P2)-hybrid $[(P_1 \times P_2)F_1]$ combination. It was hoped that such a comparative study would produce data bearing on the importance of nonimmunological factors in the interpretation of the poor growth phenomenon.

Erythropoiesis, measured by 24-hr ^{59}Fe uptake by spleens and erythrocytes, was studied in mice of several genotypes one to two weeks after irradiation and transplantation of bone marrow cells from isogenic or allogenic mice.

Results. – When two parental strains, B6 and D2, and their F_1 hybrid offspring were compared in the nine possible donor-host combinations, erythropoiesis of three groups ($F_1 \longrightarrow F_1$, B6 $\longrightarrow$ B6, and D2 $\longrightarrow F_1$) was significantly greater, and of three others (B6 $\longrightarrow$ D2, B6 $\longrightarrow F_1$, and D2 $\longrightarrow$ B6) significantly less than that of the remaining groups, as shown in Table 14.5.1. B6 and D2 marrow tested separately in recipients of several other genotypes gave rise to variable erythropoiesis, the extent of which could not be simply correlated with *H-2* homozygosity or type of the recipient. Marrow from male and female donors produced erythrocytes equally well in recipients of either sex.

The data show that growth of cellular transplants in an irradiated host cannot be predicted accurately from knowledge of *H-2* disparities alone. They do not offer any support for an immunological interpretation of poor growth of parental cells in F_1 hybrids.

14.6 ATTEMPTS TO IMMUNIZE F_1 HYBRID MICE AGAINST THEIR PARENTS

Joan Wright Goodman Gayle C. Bosma

Introduction. – If an immunological interpretation of poor growth of parental cells in hybrids is

Table 14.5.1. Uptake of $^{59}Fe^a$ by Erythrocytes and Spleens of Chimeras

Donor of 10^6 Bone Marrow Cells	Recipient of Marrow	Number of Mice Assayed[b]	Mean ^{59}Fe Uptake (% of injected dose) by	
			Erythrocytes	Spleens
B6D2F_1	B6D2F_1	44	29.05 ± 1.59[c]	9.62 ± 0.42
B6	B6	44	22.96 ± 1.23	11.74 ± 0.41
D2	B6D2F_1	44	21.33 ± 1.67	9.28 ± 0.38
B6D2F_1	D2	38	18.42 ± 1.07	6.51 ± 0.36
D2	D2	42	12.27 ± 0.98	5.89 ± 0.32
B6D2F_1	B6	46	11.92 ± 1.05	7.81 ± 0.49
B6	D2	46	2.78 ± 0.51	1.59 ± 0.24
B6	B6D2F_1	45	1.38 ± 0.15	0.57 ± 0.07
D2	B6	41	1.30 ± 0.36	1.06 ± 0.24

[a]24-hr uptake; ^{59}Fe injected seven days after marrow.

[b]Sum of four separate experiments in each case.

[c]Standard error of the mean.

Table 14.6.1. Effect of Preimmunization on Erythropoiesis in Radiation Chimeras

Experiment	900-r Irradiated Host	Preimmunized[a] to Spleen Cells	Marrow Donor (millions of cells)	Number of Mice	24-hr ^{59}Fe Uptake (% of injected dose)[b] by Erythrocytes
A	$B6D2F_1$		$SCAF_1$ (11.3)	12	46.63 ± 3.21
		$SCAF_1$		6	0.02 ± 0.00
B	B6	B6	A. BY (2.0)	38	22.67 ± 1.02
		A. BY		39	5.68 ± 2.02
C	$B6D2F_1$	B6	B6 (10)	40	30.66 ± 1.54
		D2		40	22.25 ± 1.90
		F_1		39	34.61 ± 2.41
D	$B6D2F_1$	B6	D2 (3)	22	12.46 ± 1.17
		D2		19	30.96 ± 4.68
		F_1		21	30.93 ± 2.42
E	$B6D2F_1$	B6 3×10^7, viable	D2 (3.5)	39	28.50 ± 1.78
		B6 3×10^7, dead[c]		40	40.74 ± 1.59
		B6 3×10^8, dead[c]		40	38.67 ± 2.13

[a] 3×10^7 viable cells, unless otherwise noted, given intraperitoneally ten days prior to irradiation.
[b] 0.5 μc of ^{59}Fe as citrate given intravenously six days after marrow transplantation.
[c] These cells exposed to 10,000 r in vitro.

correct, then it should be possible to immunize hybrids against the antigen(s) hypothetically present in the parental strain but lacking from the hybrid offspring. Experiments were designed to test the hypothesis by active immunization, ten days prior to irradiation, of $B6D2F_1$ mice against spleen cells from the parental strains, B6 or D2, or from control, isologous hybrids. Effectiveness of preimmunization of hybrids was measured one week after irradiation by growth of a marrow transplant. This was assessed by ^{59}Fe uptake by erythrocytes of chimeras.

Results and Conclusions. – Effectiveness of preimmunization with nonisologous spleen with *H-2* or non-*H-2* disparities is shown by experiments A and B of Table 14.6.1. Experiment C shows the ineffectiveness of B6 to preimmunize against B6. D2 cells, however, were able to reduce erythropoiesis by transplanted B6 marrow. Experiment D shows a similar deleterious effect of preimmunizing B6 cells when D2 marrow was transplanted. That preimmunizing cells must be viable to produce this effect is shown by experiment E.

From these data it appears that there are no parent-specific antigens in either B6 or D2 mice against which $B6D2F_1$ mice can be immunized. When living spleen cells are inoculated into hybrids before irradiation and marrow transplantation, they are able to reduce the effect of a homologous transplant. It is possible that this represents a secondary response of the inoculated spleen cells (B6 against D2 and D2 against B6) that survived 900 r.

14.7 HISTOLOGIC STUDY OF GROWTH OF PARENTAL BONE MARROW IN F_1 HYBRID MICE

Joan Wright Goodman Fay B. Martin
C. C Congdon

Introduction. – In some types of F_1 hybrid mice, cells of one parental type do not grow as well as cells from the other parent or its own F_1.[1] This finding is quite unexpected from a genetic point of view, and many investigators have tried to explain the phenomenon. Demonstration of the phenomenon has been made by tumor cell growth

and by hemopoietic cell transplantation in irradiated F_1 hybrid mice. In the latter situation the differential poor growth of one parent's cells compared with cells of the other parent has been shown primarily by isotope techniques. The present histologic study was carried out to determine the morphologic status of bone marrow cells from parental-type donors transplanted in the F_1 hybrid.

Methods. – Different groups of $B6D2F_1$ mice were given 900 r of x irradiation followed by intravenous injection of 10 million bone marrow cells from B6, D2, or $B6D2F_1$ donors. X-ray control and normal mice were also included in the study. Approximately five animals per day from each group were sacrificed and autopsied for six days after start of the experiment. Thereafter, animals were examined at 8, 10, 17, 24, and 31 days. The x-ray control group was not examined after the sixth day. Sections of hemopoietic tissues and other organs were prepared for histologic study.

Results. – The irradiated spleen in mice receiving B6 cells showed greatly retarded growth of the transplant during the first few days after intravenous injection. The F_1 hybrid cells gave many proliferating cell nests in the spleen at day 4, whereas the B6 transplant was barely discernible. Although the extent of proliferation of D2 cells was comparable to that of F_1 hybrid cells, there was more variation from animal to animal with D2 cells.

In the 8- and 10-day material, B6 cells had essentially caught up with the F_1 and D2 groups, and the differential growth was no longer seen histologically. There was no histologic evidence of a residual immune response in the F_1 mice given 10 million B6 cells. In the later intervals of the experiment immune reactions, presumably graft vs host, were present in the lymphatic tissues of the irradiated groups receiving parental cells.

Discussion. – These results confirm, by use of a histologic method, the well-established finding of poor parental cell growth in certain F_1 hybrids. The data show that slow initial transplantation takes place four to eight days after administration of cells. Evidence for a residual immune response to retard the initial growth of the transplant in the F_1 was lacking.

References

[1]See sect. 14.6.

14.8 ERYTHROPOIETIC STRESS AND STEM CELL PRODUCTION

Henrianne G. Willard

Introduction. – Homeostasis in the normal erythropoietic compartment of the mouse may be disrupted by stress such as bleeding or plethora. In the former case, the animal is made erythropenic, and compensation is made by an increase in erythrocyte production within a short time after stress is applied. When the mouse is made polycythemic by plethora, production of erythrocytes is reduced, and the erythropoietic compartment appears to remain quiescent. The object of the present series of experiments was to assess erythropoietic activity of the progenitor compartment in spleens of mice rendered erythropenic by bleeding or polycythemic by plethora.

Materials and Methods. – Female (C3H/Cum × C57BL/Cum)F_1 mice 12 to 16 weeks of age were used as donors and recipients. Mice were made polycythemic by two successive daily injections of 1 ml of whole blood and were used as donors at various intervals after the second plethora. Other donor mice were made erythropenic by bleeding (about 35% of blood volume). Spleens from two donors were pooled for each time interval. Spleen cell suspensions were prepared, counted, and diluted with Tyrode's solution to a concentration of 3×10^6 cells/ml. Recipient mice received 850 r of whole-body x irradiation 24 hr prior to injection of spleen cells. After eight days these mice were injected with 0.1 μc of ^{59}Fe and were sacrificed 6 hr later. Iron-59 activity in the spleen was calculated as percent of injected counts.

Results and Discussion. – Table 14.8.1 shows the average ^{59}Fe uptake of spleens from lethally irradiated mice eight days after injection of 3×10^6 spleen cells from polycythemic or erythropenic donors.

When spleen suspensions from polycythemic mice are injected into lethally irradiated recipients, the erythropoietic capacity of the injected cells is higher than the normal control value. In erythropenic mice, there is an initial drop in erythropoietic capacity, followed by an increase

Table 14.8.1. Erythropoietic Capacity of Spleen Cell Suspensions Obtained from Polycythemic Mice at Various Times After Treatment[a]

	Time After Treatment								
	0	3 hr	6 hr	1 day	2 days	3 days	4 days	5 days	6 days
Polycythemic	13.41 (14)			22.1 (20)	25.75 (20)		27.7 (10)	23.9 (10)	24.6 (8)
Erythropenic	13.41 (14)	13.3 (17)	9.76 (16)	22.0 (9)	25.99 (9)	24.72 (5)	28.6 (8)	23.9 (9)	11.03 (19)

[a]Values in parentheses represent the number of mice per point.

and overshoot after 24 hr. Values for plethorized and bled spleens one to six days after treatment are about the same and are significantly higher than the control; although in donor mice, responses to the two stimuli are opposite (increased RBC production in bled mice and decreased RBC production in polycythemic mice). These results suggest that after bleeding, the stem cell pool of the erythropoietic compartment is first depleted and then increased. This increase probably reflects an overshoot in stem cell production as a means of rapidly restoring the erythron. On the other hand, there is no ready explanation for increased stem cell production by the plethoric donor. Despite the fact that erythrocyte production is virtually nil, perhaps stem cell replication continues at a normal rate for a short time. Since few, if any, stem cells proceed into the differentiating compartment, they would accumulate.

14.9 ANIMAL TESTING PROGRAM

M. L. Simmons

Bacteriological Control. – The present bacteriological testing program includes routine fecal composite sample tests on each cage of incoming animals. These animals are tested for *Pseudomonas aeruginosa* (pigmented and nonpigmented) and *Salmonella* sp. A diagnostic service is also available for culturing lesions or suspected animals for these as well as other pathogenic organisms. *Mycoplasma plumonis*, PPLO, of mice and rats is also tested for routinely in incoming animals. Other aspects of this part of the testing program include weekly animal sampling in the Experimental Animal Facility.

Object sampling, direct and airborne, throughout the Experimental Animal Facility and testing laboratories is done on a weekly basis for detection and control of *Pseudomonas*. This program has been effective in reducing *Pseudomonas* and *Salmonella* contamination in the animals in the Facility.

Parasitological Control. – Samples of incoming animals are checked for parasites, including external parasites (mites, lice, etc.), internal parasites (pinworms, round worms, etc.), *Hemobartinella muris*, *Eperythrozoon*, coccidiosis, and ear mites. Infected animals are treated and allowed to leave the quarantine building only when infection has been eliminated.

Genetic Control. – Each week five mice of a single inbred strain are randomly selected for routine skin grafting. The grafted animals are then held for a period of two months before results are reported. Any grafts rejected during this period would result in immediate notification of our supplier concerned and an investigation.

Virus Control. – During late September or early October of 1965, our virus-testing laboratory, under construction, will be completed. We will then be able to test for most latent murine viruses, including mouse adenovirus, reovirus type 3, Sendai, lymphocytic choreomeningitis, polyoma, GDVII, pneumonia virus of mice, K virus, mouse hepatitis virus, and possibly others. We anticipate that this program will be of great value in defining autopsy lesions of unknown pathogenicity in both experimental and stock animals.

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Participation in Cooperative Projects:
AEC-NASA Space Biology Program (11.1, 11.2)
NIH-AEC – Carcinogenesis Programs (24.8)

[a]Dual assignments.
[b]Consultant.
[c]Visiting investigator from abroad.
[d]British Empire Cancer Campaign of Great Britain.
[e]Student trainee.
[f]Loanee.
[g]Temporary summer employee.
[h]American Association of University Women.

15.1 DELAYED SOMATIC EFFECTS OF X IRRADIATION IN UTERO ON MICE

J. W. Conklin K. W. Christenberry

Introduction. – Because of the reported association between diagnostic x irradiation in utero and an increased risk of cancer in childhood,[1] this experiment was undertaken to extend studies which failed to reveal carcinogenic effects of antenatal irradiation in RF mice.[2]

Results and Discussion. – Pregnant RF female mice were exposed to 50 or 150 r whole-body x irradiation at 9½, 12½, or 17½ days of gestation. Control females were sham irradiated at comparable gestational ages. After exposure, the females were returned to their respective cages, and their pregnancies were allowed to come to term. Their litters were weaned at three weeks of age, caged by sex and treatment in groups of ten, observed thereafter throughout life, and necropsied at death.

Exposure to 50 or 150 r at the ages listed caused no significant antenatal mortality, neonatal mortality, decrease in longevity, or change in the incidence of leukemia or other major diseases (Table 15.1.1). The absence of late somatic radiation effects in this experiment is consistent with the negative results of earlier investigation on the effects of antenatal irradiation in RF mice.[2] The absence of such effects indicates that our mice are strikingly more resistant to radiation carcinogenesis and life shortening before birth than when exposed after birth. The significance of these results for man, however, may be complicated by the absence of neoplasms in immature mice analogous to the cancers of childhood reported to be associated with irradiation in utero.[3]

References

[1]B. MacMahon and G. B. Hutchison, *Acta, Unio Intern. Contra Cancrum* (in press); see *Hearings, Subcommittee on Research, Development, and Radiation, Joint Committee on Atomic Energy, 88th Congress, Aug. 20–27, 1963,* part 2, p. 597.

Table 15.1.1. Longevity and Neoplasms in Prenatally Irradiated RF Mice

X-Ray Exposure (r)	Gestational Age at Time of Exposure (days)	Number of Mice[a]	Mean Age at Death (days)	Incidence of Neoplasms				
				Leukemia			Lung Tumor (%)	Ovarian Tumor (%)
				Myeloid (%)	Thymic Lymphoma (%)	Other Types (%)		
0	9½	167	592	5	7	32	21	9
0	12½	229	607	3	8	36	23	4
0	17½	230	556	6	4	38	20	5
50	9½	231	589	2	5	38	27	3
50	12½	228	563	2	8	38	20	4
50	17½	227	562	1	5	46	21	6
150	9½	229	610	3	6	40	26	8
150	12½	224	622	3	6	39	26	7
150	17½	229	608	2	6	32	29	4

[a]Weanling males and females in approximately equal numbers.

[2]A. C. Upton *et al.*, *Proc. Soc. Exptl. Biol. Med.* **104**, 769 (1960).

[3]A. C. Upton, *Natl. Cancer Inst. Monograph* **14**, 221 (1964).

15.2 DEPRESSION OF THE MEGAKARYOCYTE-PLATELET SYSTEM IN RATS BY TRANSFUSION OF PLATELETS

T. T. Odell, Jr. C. W. Jackson
Rebecca S. Reiter

Studies of Cronkite *et al.*[1] indicated that transfusion of large numbers of fresh platelets into rats produced a platelet depression that was apparent about 4 or 5 days later, after the transfused platelets had disappeared. The occurrence of this depression, which lasted 4 or 5 days, suggests that high levels of platelets in the circulation may inhibit platelet production. To investigate this hypothesis we have made counts of the numbers of megakaryocytes, the platelet precursors, in marrow after platelet transfusions and have attempted to assess the rate of platelet production by measuring the labeling of platelets with $Na_2{}^{35}SO_4$.

Results and Discussion. – Raising the platelet count of Sprague-Dawley rats to three times normal levels by transfusion of freshly collected platelets produced the expected depression below normal several days later, reaching its lowest point, 74% of normal, on the sixth day and then returning to normal 3 days later. When the peripheral platelet count was raised to nine times normal by transfusion of platelets on three successive days, the peripheral count declined to a low of about 30% of normal on the sixth day after the third transfusion, again returning to the normal range about 3 days later. The platelet count did not appear to oscillate during the first 12 days after returning to normal.

The number of megakaryocytes in the marrow of rats that were transfused with platelets on three successive days, raising the peripheral platelet count $3\frac{1}{2}$ hr after the third platelet transfusion to 5.5×10^6 platelets/mm^3 or more, was reduced approximately half on the third and fourth days after beginning transfusions (Table 15.2.1). When only a single platelet transfusion was given, however, little or no effect on megakaryocyte number was apparent.

When rats transfused with platelets either once or three times were subsequently injected with

Table 15.2.1. Megakaryocyte Number After Transfusion of Platelets at 0, 24, and 48 hr

Total Number of Platelets Injected	Number of Platelets/Injection	Peripheral Platelet Count[a] (platelets/mm^3)	Megakaryocytes/HPF[b] Experimental	Megakaryocytes/HPF Control	Time Marrow Sampled (days)
$\times 10^9$	$\times 10^9$	$\times 10^6$			
142.8	43.7–53.8	5.50	4.7	9.9	3
		5.51	3.5	8.1	
181.8	48.8–72.2	7.42	6.1	8.8	3
		6.10	5.2	9.2	
		7.30	5.4		
196.2	62.0–68.0	7.50	4.9	10.7	4
		7.03	6.5	10.3	
			Mean = 5.2	Mean = 9.5	

[a]The peripheral platelet count was taken about $3\frac{1}{2}$ hr after the 3rd platelet transfusion.

[b]Numbers of megakaryocytes per high-power field. From 10 to 100 fields (usually 30 or more) were counted in a section of femur of each rat. Each row of values is from one rat.

$Na_2{}^{35}SO_4$, the radioactivity of platelets collected 48 hr after isotope injection was less than that of controls transfused with a similar volume of saline instead of platelets, implying a reduced production of platelets.

All these findings, the drop below normal of the peripheral platelet count, the decline in number of megakaryocytes in the marrow, and the decreased radioactivity of platelets after transfusion of large numbers of viable platelets, indicate that supranormal numbers of platelets in the peripheral circulation may inhibit the production of blood platelets. It appears that the mechanism involves an inhibition of the maturation of premegakaryocytic cells into recognizable megakaryocytes. The inhibition is transient, since the platelet count returns to normal within a few days.

References

[1]E. P. Cronkite *et al.*, p. 595 in *Blood Platelets*, Little, Brown, Boston, 1961.

15.3 SIZE DISTRIBUTION OF BLOOD PLATELETS AFTER WHOLE-BODY X IRRADIATION

T. T. Odell, Jr. C. W. Jackson
Rebecca S. Reiter

It is well known that whole-body x irradiation damages hemopoietic tissue, with consequent changes in the numbers of circulating peripheral blood cells. In addition, the age composition of the circulating cell populations is undoubtedly altered during the phases of acute injury and recovery owing to changes in production of these cells by the hemopoietic tissue. Since it was shown that platelet size decreases with platelet age, we are studying platelet size distribution during the period of acute injury and recovery from a single exposure to a midlethal dose of x irradiation as an indicator of the effects of radiation on platelet production and survival.

Results and Discussion. – Sprague-Dawley rats were exposed to 900 r of 300-kvp 20-ma x rays with 3 mm of Al filtration at a dose rate of 66 r/min. Megakaryocytes in femoral marrow had decreased to 10% of normal values at 4 days and had practically disappeared altogether by the 6th day; after remaining low through the second week they began to increase in number but had not reached normal levels by the 24th day, when this set of experiments was terminated. The peripheral platelet count followed a similar pattern except that the curve was displaced approximately 2 days to the right of that of the megakaryocytes, that is, 2 days later.

Platelets were collected from the blood of rats killed at intervals after irradiation and were resuspended in physiological saline, and their pattern of size distribution was determined using a Coulter electronic particle counter and particle-size-distribution plotter. The mean size of platelets of irradiated rats was similar to that of untreated controls until the 7th day, when it was higher (Fig. 15.3.1). No samples were measured during the second week because of the scarcity of platelets. From the 14th through the 24th day the mean platelet size was always greater than that of controls. This was also true in another experiment in which platelet size was determined at intervals between the 16th and 30th days after irradiation (Table 15.3.1). The greater size of the platelets during the third week after radiation suggests that their average age is younger than that of controls. This is consistent with our expectation, since a new population of platelets is being produced at this time. If the older platelets are preferentially used to repair and maintain the

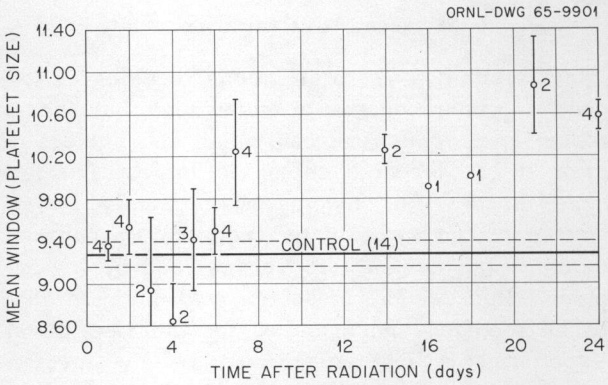

Fig. 15.3.1. Mean Platelet Size After 900 r of Whole-Body X Irradiation. Size is shown as mean window. Numbers indicate number of rats at each point, and bars denote standard errors of the means. The mean of normal (untreated) platelet populations is shown by a horizontal solid line, with the broken lines denoting the standard errors of the means.

Table 15.3.1. Platelet Size After Whole-Body Radiation (900 r)

Day After X Ray	Number of Rats	Mean Window (size)	Standard Error
0	10	8.54	0.143
16	3	9.87	0.151
18	3	9.91	0.149
21	3	9.34	0.217
25	3	9.65	0.295
30	3	9.24	0.270

vascular system, the youthfulness of the population would tend to be extended until normal numbers and normal turnover times were regained.

The larger mean platelet size on day 7 (Fig. 15.3.1) may indicate that the few platelets remaining at this time are mostly young newly produced platelets. Additional studies are needed to verify this point and to determine when the platelet-size-distribution curve returns to normal during the recovery period.

15.4 SEMIAUTOMATIC RECORDING AND COMPUTER RETRIEVAL OF MOUSE BREEDING DATA[1]

L. J. Serrano Carlene C. Amsbury

Introduction. – Accurate record keeping is essential in the production of inbred mice, but manual record keeping is time consuming and subject to error if the breeding colony is large. Furthermore, hand-written records must usually be rewritten on work sheets for transferal to punched cards if the data are to be analyzed by a computer. Because the production and pedigree data to be recorded and analyzed for the Low Level Experiment[2] will be voluminous, we have investigated electronic systems for mechanically and semiautomatically preparing the data for computer use. We have sought, in general, a system to simplify and standardize data handling and to reduce the number of errors inherent in the process. Specifically, our objectives are the following: (1) elimination of the need for manual ledger recording, (2) preparation of the information for computer input, with a minimum of intermediate steps between observation and data processing, (3) maintenance of maximum flexibility in collecting and retrieving information, (4) use of English terminology, with elimination of codes insofar as possible, (5) development of a system easily learned and used, (6) standardization of data recording to avoid differences due to different workers, and (7) use of recording equipment in breeding rooms that would be economical in space requirements, easily moved by one man, and able to withstand occasional sterilization by ethylene oxide gas.

Results and Discussion. – In the fall of 1963 after comparing several commercially available "data collection systems," we selected the IBM 357 system for trial mainly because it offered the largest variety of methods for putting data into the system and the greatest availability of assistance from the manufacturer. This equipment consists of output stations located in the animal rooms that are connected by cable to an automatic card punch machine located in the biometrics area. The data are put into output stations with the use of prepunched cards ($3\frac{1}{4} \times 7\frac{1}{2}$ in.) or badges ($2\frac{1}{4} \times 3\frac{1}{4}$ in.), and a manual entry station which enters variable data such as weight, sex, and number born or weaned. The remote automatic card punch machine converts the electrical impulses transmitted from the output station into holes in standard Hollerith cards for input into the computer. Error controls have been incorporated in the system that warn an operator recording a transaction improperly, thereby preventing such errors from entering the records. Several computer programs have been developed to record and summarize the data, to instruct breeding operations, and to store the information in computer language on tape so that it can be quickly retrieved and analyzed. Weekly lists are routinely prepared by the computer which designate the mice to be mated, weaned, retired, or placed on experiment, and which identify good and poor breeders, etc.

The system, which has been in use since July 1964, is still being continuously improved, but its accuracy is now at least equal to that of our manual records, and it stores the information in a much more accessible form. It would appear to have promise, therefore, not only for use in the Low-Level Experiment but in animal breeding colonies generally.

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15.5 SEX-LIMITED HYBRID RESISTANCE TO PARENTAL MARROW GRAFTS

Gustavo Cudkowicz Lou C. Satterfield

Introduction. – Marrow cells of DBA/2 and B10.D2 donor mice grow deficiently on transplantation into male F_1 hybrids from certain crosses but not into their female siblings.[1] The experiment to be described in the following was undertaken (1) to establish whether the resistance exhibited by such male F_1 hybrids toward parental marrow grafts is transmitted to segregating generations of mice, and (2) to discriminate between sex-linked and sex-limited inheritance.

Results. – B10.D2 ♀ marrow (5×10^5 cells) was transplanted into x-irradiated (700 r) recipients, and its growth was evaluated five days later by measuring the uptake of ^{131}IUdR in recipient spleens. Marrow was given to (B10 ♀ × B10.D2 ♂)F_1 hybrid mice of both sexes and to backcross progeny mice. The backcrosses were of two classes: In [(B10 ♀ × B10.D2 ♂)F_1 ♀ × B10.D2 ♂]BC mice, half the males resemble the B10.D2 parent with respect to sex-chromosome constitution, possessing both sex chromosomes of B10.D2 origin; the other half resemble the F_1 mice by possessing sex chromosomes of B10 ♀ and B10.D2 ♂ origin. In [B10.D2 ♀ × (B10.D2 ♀ × B10 ♂)F_1]BC, all male mice possess sex chromosomes of different origin, that is, from B10.D2 ♀ and B10 ♂ parents. The grafted B10.D2 marrow grew deficiently in F_1 males and in approximately half the backcross male mice of either class (Table 15.5.1).

Conclusion. – Resistance and susceptibility to B10.D2 marrow grafts segregated in backcross male mice at 1:1 ratio. The resistance was, furthermore, independent of the sex-chromosome assortment of the backcross male mice. These findings indicate that the resistance of F_1 hybrid males from B10 and B10.D2 parents toward B10.D2 marrow is controlled by one pair of autosomal alleles and that the expression of resistance is sex limited.

Table 15.5.1. Mean Splenic Uptake of ^{131}IUdR in Recipient Mice Following Transplantation of 5×10^5 B10.D2 Marrow Cells

Recipient Strain	Sex	Number	Uptake of ^{131}IUdR (% ± standard error)
(B10 ♀ × B10.D2 ♂)F_1	♀	39	0.60 ± 0.03
	♂	34	0.19 ± 0.02
[(B10 ♀ × B10.D2 ♂)F_1 ♀ × B10.D2 ♂]BC	♀	50	0.71 ± 0.04
	♂	27	0.12 ± 0.01
	♂	27	0.59 ± 0.04
[B10.D2 ♀ × (B10.D2 ♀ × B10 ♂)F_1 ♂]BC	♀	83	0.57 ± 0.03
	♂	32	0.11 ± 0.007
	♂	38	0.55 ± 0.03

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15.6 LINKAGE STUDIES BETWEEN THE HYBRID RESISTANCE FACTOR AND THE DETERMINANT OF THE THYMIC-LEUKEMIA (TL) ANTIGEN[1]

Gustavo Cudkowicz Lou C. Satterfield

Introduction. – Inbred $H\text{-}2^b$ mice possess in the D region of the complex *H-2* locus a genetic determinant the expression of which requires homozygosity. F_1 hybrid mice heterozygous for this determinant are incompatible for transplanted parental $H\text{-}2^b$ hematopoietic cells.[2,3] It is not known at present whether the hybrid resistance factor lies within the D region of *H-2* in linkage group IX or outside the *H-2* locus. The discovery that the determinant of the TL antigen is situated one crossover unit away from the D end of *H-2* provided the opportunity for further linkage studies.

Results. – C57BL/6 ♀ marrow (10^6 nucleated cells) was transplanted into isogenic, (C57BL/6 × A)F_1, and segregating backcross progeny mice of both sexes. The latter mice were offspring of TL+/TL– $H\text{-}2^b/H\text{-}2^b$ females backcrossed to C57BL/6 TL–/TL– $H\text{-}2^b/H\text{-}2^b$ male mice. Five days after transplantation the extent of marrow growth was estimated in the recipient spleens by the ^{131}IUdR uptake method. The TL phenotype of the backcross mice was determined by serological (cytotoxic) tests performed on thymic biopsies.[4] C57BL/6 marrow grafts grew deficiently in the F_1 hybrid recipients but grew without inhibition in either class of backcross mice segregating for TL (Table 15.6.1).

Table 15.6.1. Splenic Uptake of ^{131}IUdR in Recipient Mice Following Transplantation of 10^6 C57BL/6 Marrow Cells

Recipient Strain	TL Phenotype	Number	Uptake of ^{131}IUdR (% ± standard error)
C57BL/6	TL–/TL–	10	0.31 ± 0.02
(C57BL/6 × A)F_1	TL–/TL+	10	0.02 ± 0.001
Backcrosses	TL–/TL+	16	0.27 ± 0.01
	TL–/TL–	14	0.28 ± 0.02

Conclusion. – Backcross mice segregating for the TL antigen were uniformly susceptible to C57BL/6 parental marrow grafts. This indicated that the genetic determinant of hybrid resistance is different from the determinant of TL and closely linked with the D region of *H-2*. The experiment did not, however, provide information on the sequence of TL, hybrid resistance, and components of the D region of *H-2*.

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[1]In cooperation with E. A. Boyse and L. J. Old, Department of Immunology, Sloan-Kettering Institute for Cancer Research, New York.

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15.7 GROWTH OF TRANSPLANTED MOUSE BONE MARROW AS AFFECTED BY HYPERTRANSFUSION POLYCYTHEMIA

Michael Bennett G. M. Shearer

Introduction. – Hypertransfusion of recipient mice with isogenic erythrocytes increases the rate of multiplication of transplanted marrow stem cells in one-month-old radiation chimeras.[1] By way of explanation one might postulate that, if the stem cells were pluripotent, regression of erythropoiesis might shunt some of these cells into granulocytopoiesis or megakaryocytopoiesis, thereby accelerating the chimera's hematopoietic recovery. However, experiments on early marrow cell repopulation of the spleens of polycythemic recipient mice have met with conflicting results.[2,3] In the following experiment the effects of hypertransfusion of recipient mice with isogenic erythrocytes initiated on the day of irradiation and marrow transplantation were compared with the effects of similar hypertransfusion initiated three days later.

Results and Discussion. – Adult female (C3H/Anf × C57BL)F_1 mice were exposed to 800 r of whole-body x radiation and shortly thereafter infused intravenously with 1×10^6 isogenic bone marrow cells. One group of recipients subsequently received intraperitoneal injections of packed isogenic erythrocytes on days 0, 2, 4, and 6; a second group of recipients received similar erythrocyte injections on days 3, 4, and 6. A third control group was injected with Tyrode's solution. Mice exposed to x radiation but not grafted with bone marrow cells served as radiation controls and received either erythrocytes or Tyrode's solution as described. All prospective blood donors were exposed to 1000 r of x radiation to inactivate circulating stem cells. Growth of the transplanted marrow in the spleens of recipients was estimated by measuring the uptake of ^{131}I-iododeoxyuridine (^{131}IUdR).

The growth of the transplanted marrow was exponential over a certain period of time, as indicated by the ^{131}IUdR uptake values (Fig. 15.7.1).

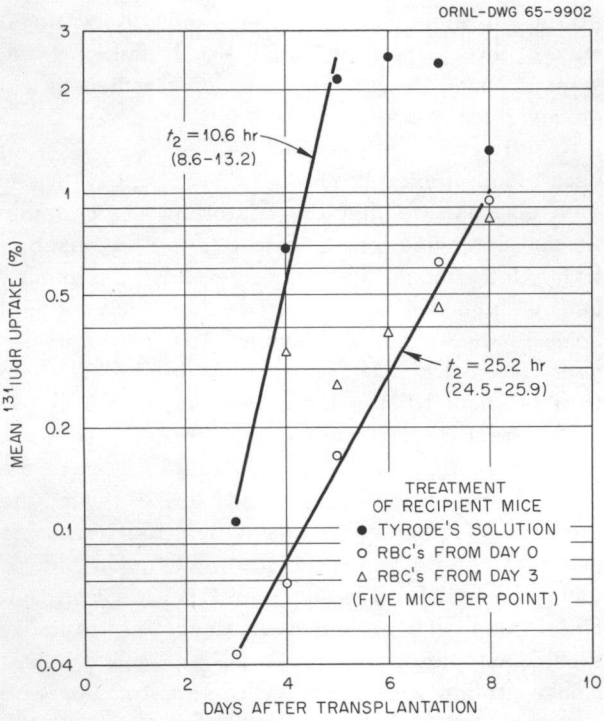

Fig. 15.7.1. Growth of DNA-Synthesizing Cells in the Spleen of Normal and Polycythemic Mice Infused with 10^6 Isogenic Marrow Cells.

The relative size of the proliferating cell pool appeared to be smaller in the polycythemic mice, in agreement with the results of Curry *et al.*[3] Furthermore, the cells repopulating polycythemic recipients did not incorporate ^{59}Fe, and no recognizable erythroid cells were seen in microscopic preparations of their spleens. When red blood cells were transfused three days after marrow transplantation, the growth of the DNA-synthesizing cell population in the recipient spleens slowed within 24 hr, presumably because erythropoiesis was suppressed, but subsequently became indistinguishable from the growth curve in mice made polycythemic on the day of marrow grafting (Fig. 15.7.1). Hence, no evidence was obtained for the shunting of pluripotent undifferentiated precursor cells into leukocytopoiesis upon short-term hypertransfusion-induced polycythemia. The multiplication of nonerythropoietic cells in the spleens of marrow recipients was, moreover, not detectably affected by the full erythropoietic activity going on during the three days prior to hypertransfusion.

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15.8 EFFECT OF DIET ON THE 30-DAY MORTALITY OF ACUTELY X-IRRADIATED GERM-FREE MICE

H. E. Walburg, Jr. Edna I. Mynatt
D. M. Robie

It has been shown that animals consuming a diet deficient in the essential nutrients are less resistant to x radiation than animals fed a complete diet.[1,2] On the other hand, supplemental feeding of conventional animals during the period of radiation anorexia has not increased survival.[3] In our experiments, a filter-sterilized liquid diet containing all known essential nutrients required by the mouse (Nutrico, purchased from Nutritional Biochemical Corporation, Cleveland) was fed ad libitum as the sole source of fluids. The mice

Table 15.8.1. Effect of Dietary Supplements on 30-day Mortality in X-Irradiated Germ-Free ICR Mice

Dose (r)	Supplement													
	Nutrico		None		Amino Acids		Fatty Acid		Carbohydrates		Salts		Vitamins	
	Number Irradiated	Percent Mortality	Number Irradiated	Percent Mortality	Number Irradiated	Percent Mortality	Number Irradiated	Percent Mortality	Number Irradiated	Percent Mortality	Number Irradiated	Percent Mortality	Number Irradiated	Percent Mortality
900	7	57												
850	40	35	41	81	10	90	9	78	35	86	10	100	23	91
800	7	29	6	100										
750	5	0	6	50										
700	5	0	7	14										

were also given access to our regular isolator diet, Purina Lab Chow 5010 C, which had been sterilized with 250°F steam. Control mice were given sterile distilled water to drink. Ten-week-old germ-free ICR mice which had received the filter-sterilized supplemental diet since five weeks of age and unsupplemented control mice were exposed to various doses of x radiation and were observed for mortality.

Results and Discussion. – Germ-free ICR mice fed the Nutrico supplement had a considerably lower mortality after x irradiation than those not fed a dietary supplement (Table 15.8.1). This "protective" effect did not appear to reside in any single group of essential nutrients, since germ-free ICR mice which received a supplement of one of the constituent groups of nutrients alone (i.e., amino acids, fatty acids, vitamins, carbohydrates, or salts) had a mortality similar to that of unsupplemented mice.

Whether the protective effect of Nutrico supplement resulted from correction of a nutritional deficiency or simply from the availability of an easily obtained highly palatable food requiring little or no digestion is presently under study.

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15.9 EFFECT OF STRAIN ON THE 30-DAY MORTALITY OF ACUTELY X-IRRADIATED GERM-FREE MICE

H. E. Walburg, Jr. Edna I. Mynatt
D. M. Robie

Introduction. – The influence of strain on acute x-ray lethality in conventionally reared mice has been demonstrated in other laboratories.[1,2] To study the influence of the microbial flora on such strain differences, germ-free and conventional mice of three strains, ICR, CF No. 1, and RFM/Un, were exposed to various doses of x radiation and observed for mortality.

Results and Discussion. – The $LD_{50/30}$ for each group of mice is shown in Fig. 15.9.1. These data demonstrate that the microflora plays a role in the determination of mouse strain radiosensitivity, for although the germ-free mice consistently had a higher $LD_{50/30}$ than their conventional counterparts, the difference in $LD_{50/30}$ between germ-free and conventional mice varied with strain from 147 r for RFM males to 34 r for the CF No. 1. Also, the $LD_{50/30}$ varied only one-half as much in the germ-free (i.e., from 715 to 789 r) as in the conventional mice (i.e., from 582 to 729 r), and the order of decreasing strain radiosensitivity was different for conventional (i.e., RFM, CF No. 1, and ICR) and for germ-free mice (i.e., CF No. 1, RFM, and ICR). Nevertheless, statistically significant variations in the $LD_{50/30}$ of different mouse strains were observed in germ-free, as well as in conventional mice, indicating that although the microflora contributes to the radiosensitivity associated with strain, other determinants are also present, possibly operating through the genetic

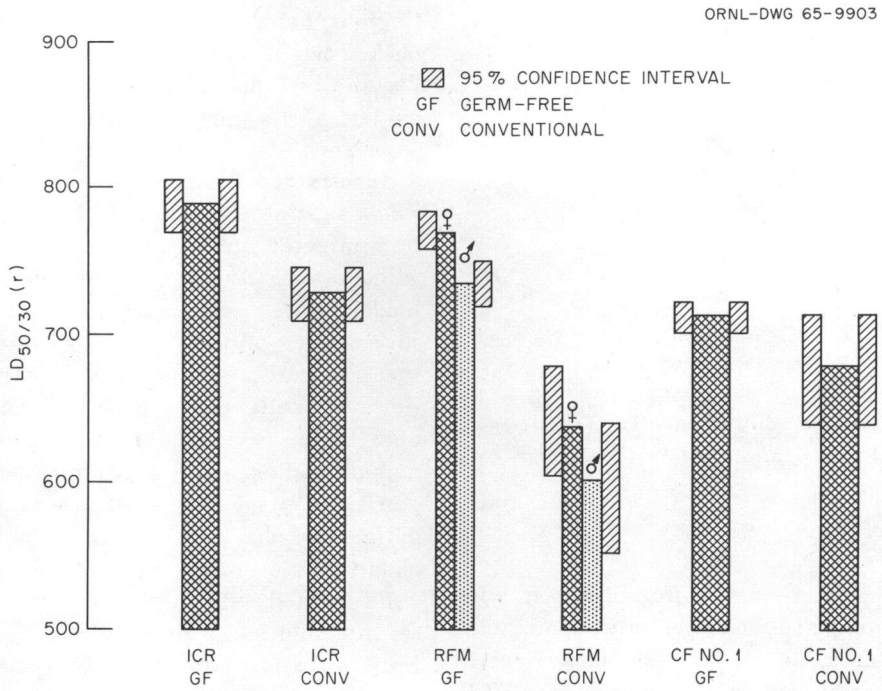

Fig. 15.9.1. $LD_{50/30}$'s of Germ-Free and Conventional X-Irradiated Mice, with the 95% Confidence Interval as Determined by Probit Analysis. Data from males and females were pooled where no significant sex difference was noted.

background. This conclusion is further reinforced by data on the survival time of lethally x-irradiated germ-free mice; one strain, RFM, had a mean survival time from three to six days less than those of the other two strains over the same dose range.

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15.10 ACTION OF A LEUKEMIA VIRUS IN SOLID TUMOR FORMATION

R. L. Tyndall J. A. Otten
N. D. Bowles Ernestine Teeter

Introduction. – A previous report described cytopathogenic effects (CPE) and transformation of cell cultures infected with the Rauscher murine leukemia virus.[1] The "crisscross" growth pattern observed in such cultures resembled that seen in cultures infected with tumor viruses.[2,3] It was of interest, therefore, to determine the relative tumorigenicity of infested, transformed cells as compared with that of uninfected, untransformed cells.

Methods and Results. – Infected and control cultures maintained on modified NCTC 109 medium were subcultured in Eagle's basal medium (4XBME). As noted previously, infected cells grown on 4XBME medium developed CPE and a transformed "crisscross" growth pattern.[1] Control cells showed no such effects when grown on 4XBME. After infected cultures had developed a marked "crisscross" growth pattern, the cells were harvested and injected subcutaneously into a group of newborn BALB/c mice at a concentration of 1×10^5 viable cells/mouse. A second group of mice were similarly injected with control, uninfected cultures. As seen in Fig. 15.10.1, the transformed cultures were highly tumorigenic, resulting in a 100% tumor incidence within 20 days after injection. Tumors induced by control cultures did not

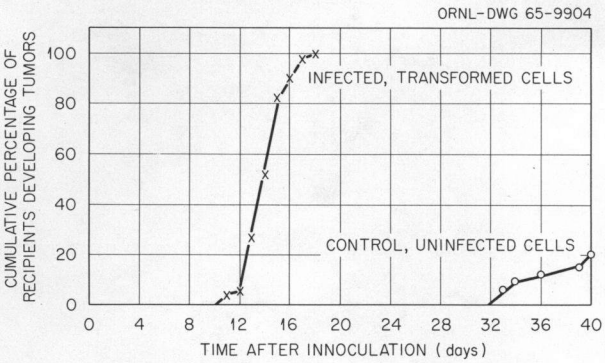

Fig. 15.10.1. Tumorigenicity of Infected, Transformed Cells as Compared with Control, Uninfected Cells.

appear until 30 or more days after injection. In addition to the increased tumorigenicity of infected, transformed cultures, 44/45 resultant tumors were classified myxofibrosarcomas, whereas 50/51 tumors induced by control cells were classified as spindle-cell sarcomas. These studies thus implicate effects of a leukemia virus in the pattern of tumorigenesis observed.

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15.11 ELECTRON-MICROSCOPIC ANALYSIS OF CELL CULTURES INFECTED WITH THE RAUSCHER MURINE LEUKEMIA VIRUS

R. F. Zeigel[1] Ernestine Teeter
R. L. Tyndall

Introduction. – A previous report described cytopathogenic effects (CPE) and transformation in cell cultures infected with Rauscher leukemia virus.[2] Development of CPE in such cultures was dependent on the type of medium in which the cells were grown. Examination of cells grown in Eagle's basal medium (4XBME) showed eosinophilic cytoplasmic inclusion bodies, cytoplasmic vacuolation, and variability in size and shape of nuclei. Cells grown in modified NCTC 109 did not show such effects.

Results and Discussion. – To determine if CPE was associated with alterations in virus distribution, infected cells grown on both media were fixed with Dalton's fixative, stained with uranyl acetate–lead citrate, and examined with the electron microscope. Virus particles were seen budding from the cell membrane in both culture conditions. However, cells grown in 4XBME and showing CPE also showed virus particles in cytoplasmic inclusion bodies. The degenerate condition of some particles suggested that they had been phagocytized by the cell. Further indication of such phagocytosis was obtained by examination of cells treated with an overlay of colloidal gold or ferritin solution. Such cells showed gold and/or ferritin particles in the same vacuoles with virus particles (Fig. 15.11.1). It thus appears that under certain conditions virus particles are concentrated in cytoplasmic vacuoles of the infected cells by phagocytosis. Whether such phagocytosis is the cause or effect of CPE and transformation remains to be determined.

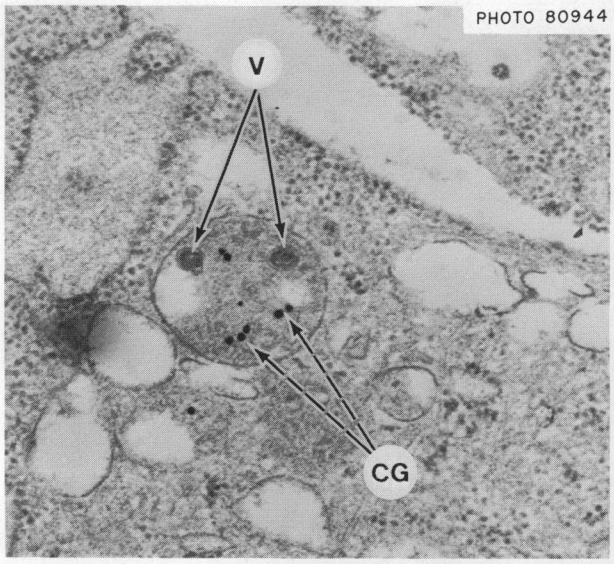

Fig. 15.11.1. Electron Micrograph of Infected Cell Showing Ingested Gold and Virus Particles. 52,500×. CG ---------> denotes colloidal gold particles; V ——> denotes virus particles.

References

[1]Laboratory of Viral Oncology, National Cancer Institute, National Institutes of Health, Bethesda, Md.

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15.12 VIROLOGICAL STATUS OF BARRIER MICE IN THE LOW-LEVEL RADIATION EXPERIMENT (LLE)

J. A. Otten Ernestine Teeter
R. L. Tyndall

Introduction. – It has been reported by Parker *et al.*[1] that "germ-free" mice are free of conventional mouse viruses such as GD VII, polyoma, PVM, K, reo. 3, Sendai, mouse hepatitis, and mouse adenoviruses. "Germ-free" RFM/Unf mice tested in our laboratory were also found to be free of these viruses. Since the mice being used in the LLE[2] are descendants of the "germ-free" RFM/Unf mice, it is important to monitor the virological status of the LLE mice continuously as one measure of the effectiveness of the barrier techniques being used to prevent their contamination.

Results and Discussion. – The presence of conventional mouse viruses is detected by the appearance of antibodies in the serum of infected mice. Antibodies against GD VII, polyoma, PVM, K, reo. 3, and Sendai are detected by the hemagglutination-inhibition (HI) test. Antibodies against hepatitis virus and adenovirus are detected by the complement-fixation (CF) test. All tests are performed with microtiter equipment. Sera from 45 LLE mice and 19 "germ-free" mice were tested for all eight aforementioned viruses. Ten additional "germ-free" mice were checked for polyoma. All the 522 tests were negative. As far as can be determined, therefore, the colony of LLE mice has remained free of the conventional mouse viruses since its establishment more than one year ago.

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15.13 USE OF IBM DATA-PROCESSING CARDS FOR FILM-MOUNTED HISTOLOGIC SECTIONS

W. D. Gude D. A. Jones

Introduction. – Pickett and Sommer[1] reported a method for mounting microscopic sections on 35-mm film as a substitute for the conventional glass slides. In a later publication[2] they indicated several improvements which resulted in more rapid and more efficient handling of the sections, permitting the method to be applied to routine histologic procedures involving a large volume of material. Since IBM cards are used in this laboratory for recording of data pertaining to experimental laboratory animals (i.e., animal history, physical examination, treatment, autopsy findings, and other results), use of the MMM5062 *Film Sort* brand aperture card (Minnesota Mining and Manufacturing Company, St. Paul, Minnesota), which contains a window approximately 1.4 × 1.2 in. cut out of the right center portion, was investigated as a means for storage of histologic sections mounted in film.

Results and Discussion. – Permanent identification of each section is made on the film with india ink as it is mounted. The film containing the mounted section can then be passed through all the usual fluids used in histologic staining with no alteration or loss of its identifying number. As shown in Fig. 15.13.1, the film containing the stained sections is readily mounted in the card window, thus permitting the sections to be filed in the same way as other data pertaining to a specific animal. Passage of the card repeatedly through the computer has failed to injure the sections. Although a few superficial scratches have occasionally been made on the film by brushes in the mechanism, these have not been noticeable at the level of the section. Moreover, the sections may be readily examined microscopically without removing them from the card.

The use of this method for mounting sections promises to economize greatly on the time, effort, and space required for filing histologic case material and thus to expedite the storage and retrieval of such material.

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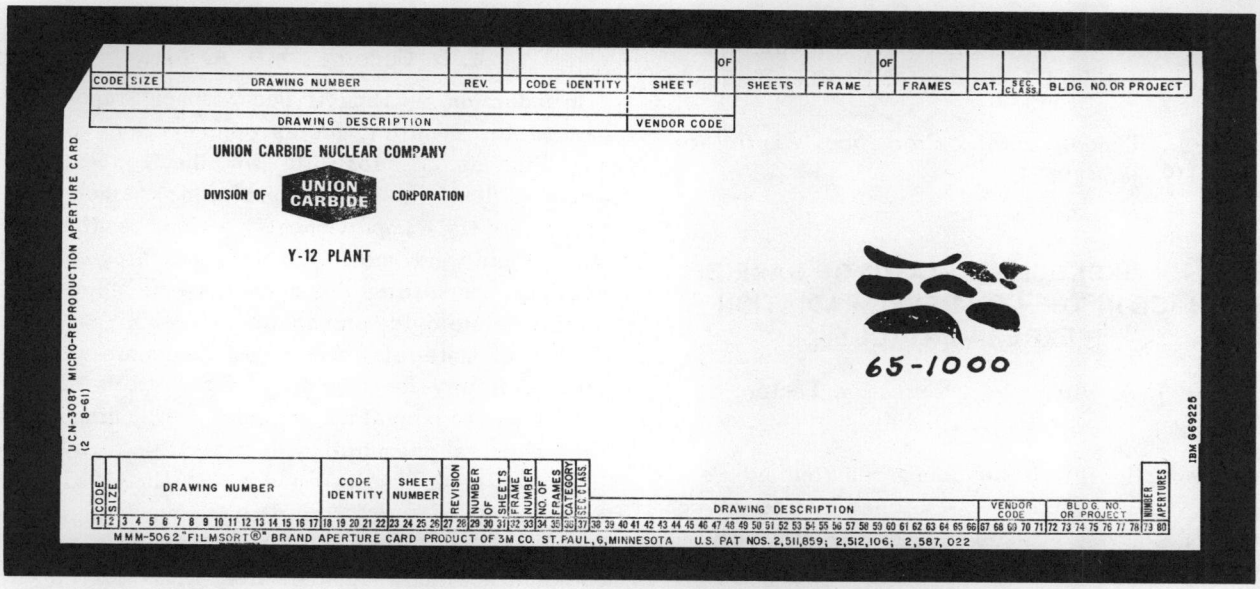

Fig. 15.13.1. IBM Card with Aperture Containing Film-Mounted Hematoxylin- and Eosin-Stained Histologic Sections.

Subcellular and Viral Physiology

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16.1 ISOLATION OF MOUSE α-CRYSTALLINE LENS PROTEIN

W. D. Fisher Chinami Takata[1]

Introduction. – Sucrose density gradient centrifugation in the B-IV zonal ultracentrifuge has been successfully employed for isolation of rat macroglobulin.[2] Since α-crystalline lens proteins are similar in size to macroglobulins, we have utilized the B-IV centrifuge for the isolation of lens protein from the mouse.

Methods. – Lenses (500) from $BC3F_1$/Cum mice were homogenized in 28 ml of 0.005 *M* phosphate buffer, pH 7.0. The homogenate was centrifuged for 30 min at 40,000 × *g*.

The supernatant (27 ml) was separated on a 1-liter 10 to 20% sucrose gradient containing 0.2 μ Miller-Goldner buffer, pH 7.5, by centrifugation to $G = \int_0^t \omega^2\, dt = 250 \times 10^9$. The gradient was collected in 40-ml cuts. The sample was concentrated by pressure dialysis against 0.2 μ Miller-Goldner buffer, pH 7.5.

Protein concentration was determined by the method of Lowry,[3] and sedimentation analysis was performed as described by Schachman.[4]

Results. – The absorbancy profile of the gradient is shown in Fig. 16.1.1. The leading peak contained 101 mg of protein. Analytical ultracentrifugation of the material concentrated from this peak showed a single peak with a sedimentation coefficient ($S_{20,w}$) of 19.9.

Discussion. – Preliminary results show that mouse α-crystalline lens protein can be isolated by zonal ultracentrifugation. Further studies of purity and additional physical-chemical studies are in progress.

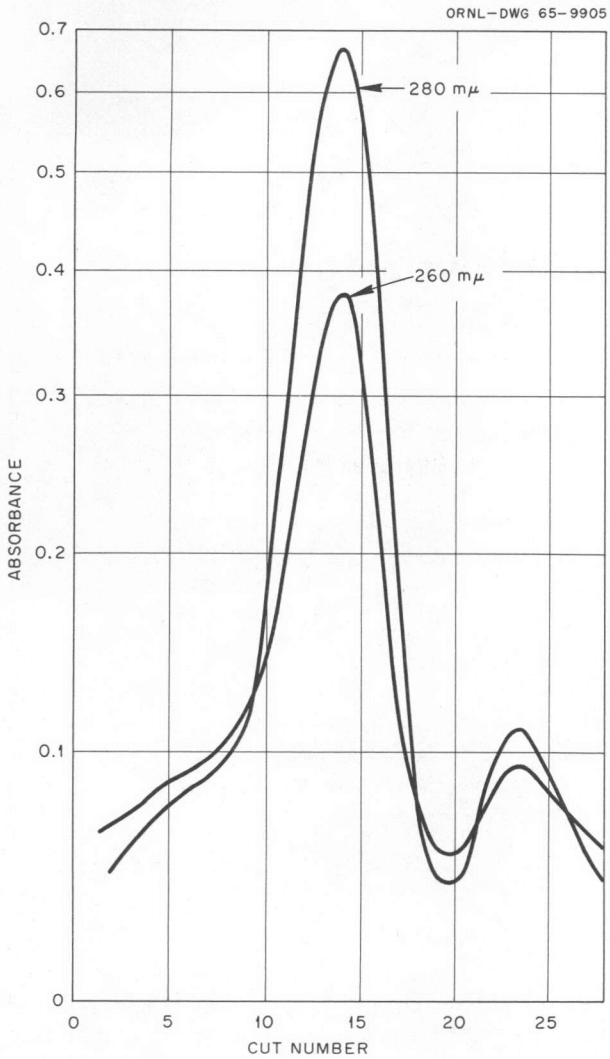

Fig. 16.1.1. Separations of Mouse α-Crystalline Lens Protein on a Sucrose Gradient. 0.2-cm light path; cut volume is 40 ml.

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Nucleic Acid Enzymology

Elliot Volkin

Microbial Systems (17.1–17.6)		Developmental Biochemistry (17.7–17.9)	
Elliot Volkin	M. Helen Jones	F. J. Finamore	Rose P. Feldman
W. E. Barnett	Dawn R. Zimmerman	Sumiko Iyeiri[b,c]	Carolyn G. Mohler[e]
R. K. Fujimura		A. H. Warner[d]	
J. X. Khym			
C. G. Mead			
J. L. Epler[a]			

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[b]Visiting investigator from abroad.
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17.1 DEOXYCYTIDINE IN PHAGE T2 DNA: A POSSIBLE SOURCE OF IN VIVO DNA BREAKAGE

Elliot Volkin M. Helen Jones

In the course of some experiments with presumably uniformly labeled phage T2 stocks, it was noticed that hydroxymethyldeoxycytidylic acid (HMC) deoxyribonucleotide was transferred to progeny DNA with slightly less efficiency than adenine, thymine, and guanine deoxyribonucleotides. This result is at variance with published data indicating uniform transfer of all four bases and HMC-glucose. Since the difference in transfer is small, a means of labeling the parental phage stocks that would amplify this effect was sought. This was accomplished by allowing only the bacterial DNA (not exogenous materials) to be the source of phage DNA labeling and collecting the very early phage produced, that is, the intracellular phage present at times about 14 to 16 min after infection. Under these conditions, the radioactivity of the dHMCP region of the column chromatogram was markedly elevated with respect to the other deoxyribonucleotides. Further separation of the various components of the dHMCP region revealed that a small amount of highly radioactive deoxycytidylic acid (dCP) is present in this fraction.

The deoxycytidine is shown to be a bona fide constituent of T2 DNA, not a contaminant from bacterial DNA, and represents perhaps 1% of the total HMC component of T2 DNA. Studies on the transfer of parental deoxyribonucleotides to progeny DNA indicate that relatively little transfer of parental dCP occurs. This and other evidence suggest that dCP residues represent unique breakage points in phage T2 DNA.

17.2 THE OLIGODEOXYRIBONUCLEOTIDE TRANSFERASE SYSTEM

C. G. Mead

The oligodeoxyribonucleotide transferase system from T2-infected *E. coli* cells is believed to be capable of condensing oligodeoxyribonucleotides with acceptor polydeoxyribonucleotides. The product of the reaction has been examined by chromatography on methylated albumin kieselguhr columns and by sucrose density gradient centrifugation. The distribution of a product from a reaction after a 1-hr incubation period when chromatographed on methylated albumin is illustrated in Fig. 17.2.1. The solid line represents the absorbance at 260 mμ due to the sonicated T2 DNA acceptor present in the reaction mixture, and the dotted line represents the radioactivity introduced as ^{32}P-labeled oligodeoxyribonucleotides that have become associated with a high-molecular-weight product. It is clear that the distribution of radioactivity does not correspond to the ultraviolet absorbancy profile but is eluted on the leading side of the peak. At zero time there is no radioactivity detected in this region, and the radioactivity that appears with time of incubation is skewed toward the leading edge even after 4 hr of incubation.

The distribution of the same 1-hr product was subjected to sucrose density gradient centrifugation and is illustrated in Fig. 17.2.2. A marker was introduced at this time consisting of tritium-labeled *E. coli* DNA to compare different incubation times and is represented as a dashed curve. It is clear that the ^{32}P label (represented by a

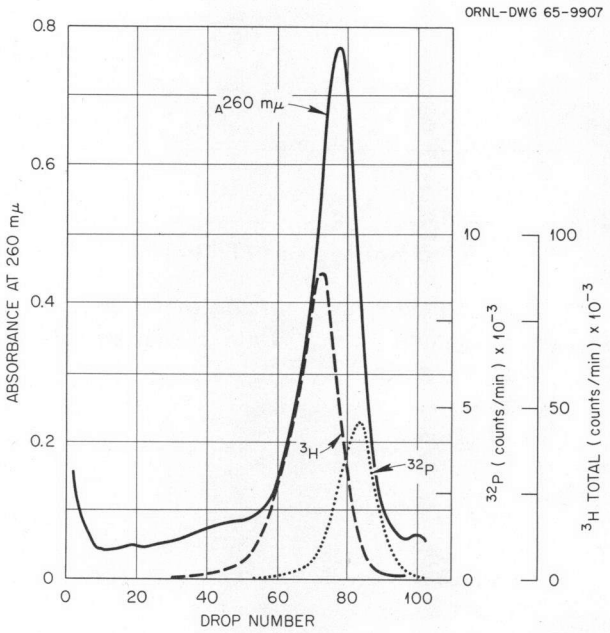

Fig. 17.2.2. Sucrose-Density-Gradient Centrifugation of Product.

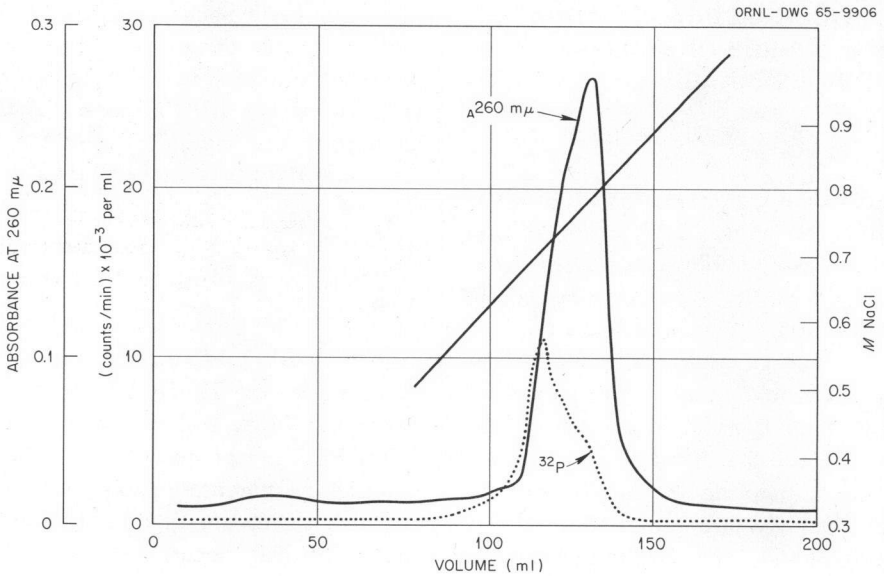

Fig. 17.2.1. Methylated Albumin Chromatography of Product.

dotted curve) derived from the labeled oligodeoxyribonucleotides is on the light side of the ultraviolet absorbance peak contributed by the sonicated T2 DNA acceptor (represented by a solid curve). Both lines of evidence lead to the conclusion that the labeled oligodeoxyribonucleotides preferentially appear in acceptor polymers of a lower molecular weight average than that of the total acceptor molecules available. Current investigations are designed to understand this phenomenon.

17.3 TWO NORMALLY OCCURRING ASPARTYL-sRNA SYNTHETASES FROM *NEUROSPORA*

W. E. Barnett J. L. Epler
Dawn R. Zimmerman

Fractionation of *Neurospora* aminoacyl-sRNA synthetases revealed the presence of two chromatographically separable aspartyl-sRNA synthetase activities. That the two activities reflect two distinct enzymes is indicated by several lines of evidence: (1) nonoverlapping DEAE chromatography profiles, (2) very different kinetics of heat inactivation, (3) only one of the enzymes aminoacylates *E. coli* sRNA with aspartic acid, and (4) apparent specificity in the aminoacylation of the different species of *Neurospora* aspartic sRNA.

17.4 R17 BACTERIOPHAGE WITH 5-FU-INCORPORATED RNA (FU-PHAGE)

R. K. Fujimura

In the previous report,[1] three questions were raised concerning the effect of 5-fluorouracil (5-FU) on phage biosynthesis. Several technical difficulties involved in the use of this analog during growth have since been overcome.

Shimura *et al.*[2] have recently reported that phage particles containing 5-FU will form only if the analog is added 17 min or later after infection. However, I have definitely obtained FU-phage by adding 5-FU at zero time of infection. The degree of inhibition of viable phage formation and the amount of ^{3}H-FU incorporated are about the same whether the 5-FU is added at 0 or 30 min after infection. Fu-phage and its RNA are being characterized further by physical-chemical and biological techniques in the hope of correlating changes in specific genetic functions of the virus with changes in biochemical structure of the viral RNA.

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17.5 CODING PROPERTIES OF ANOMALOUSLY CHARGED sRNA'S

J. L. Epler W. E. Barnett

Reports from the laboratory of Nirenberg have established that synthetic polyribonucleotide-directed ribosomal binding specificities of ^{14}C-aminoacyl-sRNA's may be employed to elucidate their coding properties and, hence, genetic codes. We have prepared heterologously charged, hybrid sRNA's and compared their binding properties with those of homologously charged aminoacyl-sRNA's relevant to this study.

Homologously Formed Aminoacyl-sRNA's. – The binding responses of ^{14}C-aminoacyl-sRNA's formed by homologous charging of *E. coli* and *Neurospora* sRNA's are shown in Table 17.5.1. The responses are specific and similar in both organisms and agree with the triplet code assignments previously shown with *E. coli* cell extracts: that is, phenylalanine, UUU; alanine, UCG; and valine, GUU.

Heterologously Formed Aminoacyl-sRNA's. – Since the *Neurospora* synthetases attach phenylalanine to three *E. coli* sRNA's, the method of Khym was used to prepare two fractions containing separated sRNA's: (1) an aqueous fraction, enriched with alanine and valine sRNA's, free of phenylalanine sRNA; and (2) an organic fraction, enriched with phenylalanine sRNA, free of alanine and valine sRNA's.

The binding responses of these heterologously charged or hybrid sRNA's are given in Table 17.5.1. PHE-sRNA$^{phe}_{coli}$ maintains the property of the homologous sRNA's and responds to poly-U and U-containing heteropolymers. However, the hybrid PHE-sRNA$^{ala+val}_{coli}$ no longer responds to

Table 17.5.1. Polynucleotide Specificity

Synthetase	^{14}C-Aminoacyl-sRNA Formed	^{14}C-Aminoacyl-sRNA Bound to Ribosomes ($\mu\mu$moles)			
		None	Poly-U	Poly-UCG	Poly-UG
E. coli (homologous)	PHE-sRNA$^{phe}_{coli}$	1.35	12.39	1.88	1.74
	ALA-sRNA$^{ala}_{coli}$	0.37	0.24	3.27	0.31
	VAL-sRNA$^{val}_{coli}$	0.15	0.11	0.27	0.71
Neurospora (homologous)	PHE-sRNA$^{phe}_{neur}$	1.51	11.03	2.14	1.76
	ALA-sRNA$^{ala}_{neur}$	0.34	0.21	0.71	0.29
	VAL-sRNA$^{val}_{neur}$	0.34	0.18	0.36	1.14
Neurospora (heterologous)	PHE-sRNA$^{phe}_{coli}$	0.20	3.06	0.37	0.29
	PHE-sRNA$^{ala+val}_{coli}$	0.42	0.39	2.66	0.46
	PHE-sRNA$^{val}_{coli}$	0.13	0.14	0.47	0.67

poly-U, but maintains the coding properties of sRNA$^{ala}_{coli}$, responding to poly-UCG. Since the *Neurospora* synthetases also attach phenylalanine to sRNA$^{val}_{coli}$, a slight response to poly-UG is also seen.

In order to verify and magnify this poly-UG response, periodate oxidation of the aqueous fraction precharged with L-valine was carried out and the valine specific sRNA obtained. The last line of Table 17.5.1 shows the increased binding response of PHE-sRNA$^{val}_{coli}$ to poly-UG.

17.6 SEPARATION OF TRANSFER RIBONUCLEIC ACIDS BY LIQUID-LIQUID PARTITION METHODS

J. X. Khym

Introduction. – Transfer RNA (tRNA) is separated into definite groups using single-batch extraction techniques by which the various amino acid acceptor RNA's are selectively partitioned between a quaternary amine dissolved in CCl_2F-$CClF_2$ and aqueous NaCl solutions.[1] Experiments are now being designed to exploit the large differences in extraction coefficients that have been found for certain groups of tRNA's and also to seek new experimental conditions that may be used to separate members within a given group. Examples of these approaches are: (1) cascade extractions where a given single organic phase containing tRNA is successively extracted with a series of fresh aqueous phases; (2) cascade extractions using a dilute concentration of amine ($\sim$0.01 *M*) in organic solvent and extracting this same organic phase repeatedly with fresh aqueous solution at a fixed concentration of salt and containing tRNA at saturation concentrations; and (3) changes in the experimental conditions of the original extraction method,[1] such as pH, and the use of salts in the aqueous phase other than NaCl.

Experimental. – Extractions were carried out as previously described.[1] In an example of the cascade type of extractions, tRNA initially at 156 units/ml (absorbance at 260 mμ) in the organic phase was extracted batchwise four times with 0.30 *M* NaCl. The same number of extractions were carried out with 0.32 *M* and 0.34 *M* NaCl solutions, and finally the tRNA that remained in the organic phase was removed by a single extraction with 0.5 *M* NaCl. An aliquot of the initial organic phase was also extracted with 0.5 *M* NaCl; this served as a control. The absorbance at 260 mμ of each aqueous fraction obtained is given in Table 17.6.1.

In studies to determine if NaCl could be replaced with other salts and still have effective partitioning of tRNA between the organic and aqueous

Table 17.6.1. Optical Density of Fractions Obtained by Cascade-Type Extractions

Aqueous Fraction	Molarity of NaCl Used	Optical Density at 260 mμ
1	0.30	24
2	0.30	22
3	0.30	4
4	0.30	1
5	0.32	14
6	0.32	20
7	0.32	4
8	0.32	3
9	0.34	17
10	0.34	6
11	0.34	4
12	0.34	5
13	0.50	23
Control	0.50	156

phases, it was found that KCl, CsCl, NH_4Cl, LiCl, and CH_3COONa were compatible with the quaternary amine dissolved in $CCl_2F\text{-}CClF_2$. Phosphate, sulfate, formate, and citrate salts gave emulsions or a double organic phase.

Results and Discussion. – Fractions 1, 2, 5, 6, 9, and 13 and the unfractionated control of Table 17.6.1 were tested singly with 12 amino acids for amino acid acceptor activity. The results given in Table 17.6.2 demonstrate that the mole composition of tRNA can be changed considerably by batch-extraction techniques. Extractions of this type would be of practical value if applied either before or after other separation procedures, such as countercurrent or column partition methods. Some of the data appear anomalous, such as a noted increase in the amounts of alanyl-, arginyl-, histidyl-, threonyl-, and leucyl-tRNA that is found in fraction 2 compared to fraction 1, in that both fractions were obtained by extracting with 0.30 *M* NaCl. The data suggest that extraction coefficients are changing with the concentration of the tRNA's or that mole composition influences the partitioning of the tRNA's.

Table 17.6.2. Changes in Percent Mole Fraction[a] of Transfer Ribonucleic Acids by Cascade-Type Extractions

tRNA Assayed	Control	0.30 *M* NaCl		0.32 *M* NaCl		0.34 *M* NaCl	0.5 *M* NaCl
		Fraction 1	Fraction 2	Fraction 5	Fraction 6	Fraction 9	Fraction 13
Glutamyl-	4.9 [1730][b]	16.1 (62)[c]	3.7 (14)	0.4 (1)	0.1 (0.4)	0.1 (0.2)	0.01 (0.02)
Lysyl-	9.7 [3430]	35.4 (68)	9.3 (18)	1.6 (2)	1.2 (2)	0.5 (0.4)	0.2 (0.1)
Valyl-	13.5 [4750]	24.6 (34)	24.8 (36)	10.3 (10)	7.4 (7)	0.4 (2)	0.8 (0.4)
Alanyl-	5.2 [1822]	5.2 (19)	10.0 (38)	7.7 (20)	3.0 (8)	1.2 (2)	0.2 (0.3)
Arginyl-	9.3 [3270]	9.0 (18)	16.3 (34)	12.6 (18)	8.3 (12)	5.6 (5)	2.0 (1)
Histidyl-	7.1 [2490]	6.3 (9)	7.8 (21)	11.0 (21)	13.1 (25)	2.8 (3)	1.0 (1)
Threonyl-	8.4 [2950]	2.8 (6)	9.0 (21)	20.4 (32)	17.8 (29)	5.6 (5)	1.9 (1)
Leucyl-	17.0 [6000]	1.7 (2)	15.0 (17)	26.4 (21)	20.8 (17)	33.9 (16)	31.0 (11)
Seryl-	7.8 [2740]	1.1 (3)	2.8 (7)	5.1 (8)	13.0 (23)	7.9 (8)	10.0 (8)
Prolyl-	6.9 [2413]	0.2 (0.4)	0.5 (2)	3.1 (6)	12.4 (25)	24.0 (28)	3.3 (3)
Tyrosyl-	4.2 [1460]	0.3 (1)	0.4 (2)	0.5 (2)	1.2 (4)	4.3 (8)	8.6 (12)
Phenylalanyl-	6.0 [2103]	0.3 (1)	0.3 (1)	0.3 (1)	0.5 (1)	9.6 (13)	39.3 (39)

[a]Figures given relate only to the 12 tRNA's tested.

[b]Values in brackets are micromicromoles per milliliter of a particular tRNA in control determined by amino acid incorporation using ^{14}C-amino acids (see ref. 1).

[c]Numbers in parentheses give the percent yield of a tRNA in a particular fraction.

Other salts that were found to substitute for NaCl give the same general type of response as to the amount of tRNA (based on absorbance measurements) found in the aqueous fraction at a given concentration of salt, as does NaCl.[1] Partition coefficients for the individual transfer RNA's using the other salt systems have not been completely determined. However, some initial data indicate that it is possible to separate phenylalanyl-tRNA from tyrosyl-tRNA when the aqueous phase contains NaCl solution buffered at pH 3.6 with acetate. These two tRNA's partition together in unbuffered NaCl solution (pH about 6.5).[1]

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17.7 NUCLEOTIDE METABOLISM DURING BRINE SHRIMP EMBRYOGENESIS

A. H. Warner F. J. Finamore

Introduction. – The observation that encysted eggs of the brine shrimp, *Artemia salina*, contain significant quantities of diguanosine tri- and tetraphosphate, while virtually no ATP, prompted us to investigate the metabolism of these unique nucleotides during brine shrimp embryogenesis in an attempt to ascertain their role in development.

Methods. – Twenty grams of dried cysts was suspended in 1 liter of ice-cold antiformic solution,[1] stirred gently for 30 min, filtered through a sintered-glass filter, and washed well with ice-cold distilled water. One-gram portions containing about 150,000 cysts were placed in each of 16 crystallizing dishes (50 × 90 mm), and the cysts were covered with 40 ml of Wardley's hatch mix containing 1000 units of penicillin per milliliter. The dishes were placed on a slowly rotating platform and kept at 30 to 31°C for the desired length of time. At 0, 2, 5, 9, 12, 18, 24, and 36 hr the contents of two dishes were pooled and filtered and washed well with ice-cold distilled water. The eggs or embryos were homogenized in ice-cold 1 *N* $HClO_4$ and centrifuged; and the supernatant fraction was deacidified by treatment with an excess of tricaprylyl amine in chloroform.[2] The perchlorate-free aqueous phase was washed once with chloroform and then applied directly to a DEAE-cellulose column (1 × 50 cm, HCO_3^- form). The nucleotides were eluted with a linear gradient of NH_4HCO_3, pH 8.6.[3]

Results. – As previously reported,[4] the acid-soluble fraction of encysted brine shrimp eggs contain the following uv-absorbing compounds in order of their elution off DEAE-cellulose: guanosine, CMP, UDP-X, AMP, an unknown, GMP, ADP, GDP, ATP, GTP, and diguanosine tri- and tetraphosphate. Analysis of these compounds during embryogenesis indicates the following:

1. Prior to emergence of the embryo from its shell (0 to 5 hr), there is a 500% increase in ATP. During subsequent development, ATP synthesis continues but at a lower rate.
2. Throughout development the content of AMP and ADP remains unchanged.
3. The diguanosine tetraphosphate content decreases slightly prior to emergence, remains constant during the emergence period (5 to 12 hr), then falls abruptly throughout the hatching period (12 to 36 hr).
4. The diguanosine triphosphate content remains constant throughout development.
5. Prior to emergence, GMP falls markedly, whereas GDP and GTP remain unchanged. At all subsequent stages all three nucleotide levels decline.

Discussion. – The ability of *Artemia* to withstand prolonged periods of desiccation and then to resume normal development upon emersion in seawater represents an interesting problem in biochemical energetics. The observation that the encysted egg contains only trace amounts of ATP compared to diguanosine tetraphosphate leads us to speculate that diguanosine tetraphosphate may be the primary source of energy for resumption of development. The initial rapid increase in ATP, apparently not at the expense of preexisting AMP or ADP, suggests either de novo synthesis or transamination of a guanosine nucleotide as a source of the newly made ATP. Also, the decrease in diguanosine tetraphosphate prior to emergence cannot be accounted for by an increase in GMP and GTP, contrary to our earlier in vitro studies.[5] Whether diguanosine tetraphosphate or its hydrolytic products are being degraded further, incorporated into acid-insoluble material, metabolized as a source of the adenosine

nucleotides, or excreted from the embryo is now under investigation.

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17.8 STUDIES ON RNA POLYMERASE FROM AMPHIBIAN EMBRYOS

Sumiko Iyeiri F. J. Finamore

In a continuing investigation of the RNA polymerase from amphibian tissues, we have recently undertaken the isolation, purification, and study of the properties of this enzyme system from developing amphibian embryos.

An active, partially purified RNA polymerase was obtained using a nuclear fraction from embryos of *Rana pipiens* in various stages of development (gastrulae, neurulae, tail bud, and hatching). Purification of the enzyme was achieved by nuclear lysis, ammonium sulfate and potassium chloride fractionation, and by DEAE-cellulose column chromatography.

The complete reaction mixtures contained 50 micromoles of Tris-HCl buffer, pH 7.6, 30 micromoles of KCl, 5 micromoles of NaF, 4 micromoles of $MnCl_2$, about 4 optical density units of amphibian liver DNA or calf thymus DNA, unlabeled ribonucleoside triphosphates, 0.8 to 1.0 micromole of ATP-^{3}H or UTP-^{3}H, and enzyme, in a final volume of about 0.7 ml. At 0 and 50 min duplicate 100-μl samples are removed and applied to filter paper disks, which are subsequently washed in ATP-TCA solution and PP_i-TCA solution and counted by a Packard scintillation counter.

Incorporation is linear with time for approximately 50 min. No incorporation of ATP-^{3}H or UTP-^{3}H into acid-insoluble products occurs in the absence of divalent metal ion or sodium fluoride and in the presence of pyrophosphates.

We have observed two different polymerase activities to be present in the nuclei of embryos: one activity occurs in nuclei before hatching, in which the presence of ribonucleoside triphosphates inhibits the incorporation of labeled precursor into an acid-insoluble product; and the other activity occurs after hatching, in which the presence of ribonucleoside triphosphates stimulates incorporation of labeled precursor into the products.

These results suggest that at least two enzyme systems may be responsible for the incorporation of labeled triphosphate into RNA during amphibian embryonic development, but only after hatching does the "normal" RNA polymerase system predominate.

17.9 IN VITRO LABELING WITH ^{32}P OF OLIGONUCLEOTIDES FROM AMPHIBIAN EGGS

F. J. Finamore Rose P. Feldman

Introduction. – In a continuing study of the oligonucleotides of amphibian eggs, we have undertaken an investigation of their metabolic activity with respect to ^{32}P incorporation in vitro.

Methods. – Mature ovarian eggs from *Rana pipiens* were incubated at 37°C for 30 min in amphibian Ringer's solution containing ^{32}P as orthophosphate. At the end of this time the eggs were washed with unlabeled medium, and the RNA was extracted by a hot-NaCl procedure.[1] The oligonucleotides were prepared and subjected to DEAE-cellulose column chromatography as described previously.[2] Two major radioactive peaks were purified by rechromatography on DEAE columns and were subjected to phosphorus analyses and enzymatic degradation procedures to ascertain their structures.

Results and Discussion. – Only two highly radioactive compounds (compounds A and B) are present in the oligonucleotide fraction from amphibian eggs after a short exposure of the eggs to ^{32}P. After purification of the compounds, analyses for total, labile, and terminal phosphorus indicated the presence of nucleoside diphosphates in both fractions. However, paper chromatography of the labeled material along with known disphosphates as reference compounds indicated that they were not identical to the common nucleoside diphosphates. Furthermore, hydrolysis of the labeled compounds with pancreatic ribonuclease, followed

either by paper or column chromatography, revealed that compound B is attacked by the enzyme. But after pancreatic ribonuclease hydrolysis of compound B, only one radioactive peak or uv-absorbing spot, similar to ATP, was found upon column or paper chromatography of the enzyme digest. Since this observation is contrary to the known specificity of pancreatic ribonuclease, we checked the digest for the presence of a non-uv-absorbing substance, such as a pyrimidine reduction product.[3] We found that, indeed, compound B contains a dihydropyrimidine component in addition to an adenine moiety.

Compound A, which is not degraded by pancreatic ribonuclease, does not contain a dihydropyrimidine and most probably represents a dinucleotide with a base sequence that is resistant to the action of pancreatic ribonuclease.

The ^{32}P label can be removed from both compounds by subjecting them to a 10-min hydrolysis at 100°C in 1 *N* H_2SO_4, suggesting that the label is incorporated into a terminal pyrophosphate linkage and not into a phosphodiester bond.

On the basis of these observations, we believe tentatively that compounds A and B are dinucleotides containing terminal pyrophosphate groups. Compound B is a dinucleotide compound of adenine and dihydropyrimidine components, and compound A is resistant to attack by pancreatic ribonuclease by virtue of its base sequence.

We are currently investigating the base sequence in compound A, the identity of the dihydropyrimidine component of compound B, and the position of the pyrophosphate groups in both compounds.

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Nucleic Acid Chemistry

Nucleotide Chemistry (18.1, 18.2)	Participation in Cooperative Projects
Waldo E. Cohn — A. Jeannine Bandy Mayo Uziel — Margaret L. Rachal[a]	NIH-AEC – Carcinogenesis Programs (24.13, 24.14)
Molecular Biology (18.3–18.5)	
F. J. Bollum[b] — Patricia N. Howard B. K. Zimmerman[b] — Janet R. McGrath Giovanni Cassani[b,c] — Patricia A. Williams J. M. Goncalves[c,d] — G. E. Houts[e]	[a]Student trainee. [b]Dual assignments. [c]Visiting investigator from abroad. [d]Fulbright Fellowship. [e]Consultant.

18.1 A 1-HR QUANTITATION OF NUCLEIC ACID COMPONENTS AT NANOMOLE LEVELS BY ELUTION ION EXCHANGE CHROMATOGRAPHY

Waldo E. Cohn — Mayo Uziel
A. Jeannine Bandy

Although the nucleic acid bases were the first biochemical substances among which the separation potential of elution chromatography on ion exchangers was demonstrated,[1–3] it was for the analysis of amino acid mixtures that automated analytical procedures were first perfected.[4] The principles utilized by the latter workers were applied to the older nucleic acid field by Anderson *et al.*,[5] resulting in a "nucleotide analyzer." Applying this machinery with such recent modifications as a scale expander to the problem of a rapid analysis of nucleic acid hydrolysates by cation exchange, we have been able to accomplish a complete analysis of the major bases or nucleosides in less than 1 hr. As little as 10 nanomoles (0.1 absorbance unit) of total material is needed, with the possibility of detecting and quantitating 1 nanomole of any separable component. These results have been obtained on 25- by 1-cm columns of commercially available resins at 1 ml/min flow rates (25–50 psi of pressure) and with 0.3-ml flow-through spectrophotometer cells. Each component is recoverable in a few milliliters of the buffer eluant. A factor of 10 in sensitivity is easily attainable with narrower columns and better-designed flow-through cells. Avoiding gradients reduces the time between analyses to a few minutes and eliminates the need for duplicate columns in sequential analyses.

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18.2 GEL FILTRATION OF OLIGONUCLEOTIDES

Mayo Uziel Waldo E. Cohn
A. Jeannine Bandy

Gel filtration provides a solution to the problem of a rapid, quantitative, and nondegradative procedure for the isolation of oligonucleotides.

A commercially available polyacrylamide gel[1] with a nominal exclusion limit of *ca.* 1600 molecular weight permits the complete removal of the salts usually encountered in the isolation of nucleotides, including mononucleotides.

Up to one-third the bed volume may be used for the sample with dinucleotides and larger molecules. However, only 10% of the bed volume is available for desalting of mononucleotides, since these are only partially excluded from the gel matrix. At flow rates of even 4 ml min^{-1} cm^{-2}, an essentially rectangular-shaped effluent profile is obtained with a sample dilution of less than 10%.

The tendency of the purine derivatives to be adsorbed by the gel matrix at acid pH values is minimized by raising the temperature and pH. At lower pH values (*ca.* 3) in the presence of only small amounts of salt, adsorption of oligonucleotides occurs. These are quantitatively recovered by washing the column with 0.1 *M* salt solution. The most effective pH range for desalting purposes is that of 7 to 9. The nucleosides and bases are not excluded from the gel matrix; instead they are retarded.

Typical contaminants that have been removed from mono- and oligonucleotides by gel filtration are urea, Na^+, K^+, NH_4^+, Cl^-, Br^-, Mg^{2+}, acetate, formate, acetic acid, and formic acid.

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18.3 PREPARATION AND PROPERTIES OF DEOXYNUCLEOTIDE POLYMERIZING ENZYMES FROM CALF THYMUS GLAND

G. E. Houts Patricia A. Williams
Janet R. McGrath F. J. Bollum

Two useful deoxynucleotide polymerizing enzymes can be isolated from calf thymus gland by a pilot-scale process devised in this laboratory. The enzymes are DNA polymerase (replicative-deoxynucleotidyl transferase) and terminal-deoxynucleotidyl transferase. These enzymes are needed in relatively large amounts for our continuing synthetic work, for kinetic studies currently under investigation, for immunological studies, and for other analytical experiments anticipated in the future.

To obtain the necessary enzyme, we have recently gone through six 30-kg preparations as previously described and find that the enzyme purification process proceeds smoothly. We have estimated the molecular weights of these two polymerizing enzymes by Sephadex G-200 filtration and find the terminal transferase to have a molecular weight of about 33,000 and the DNA polymerase about 105,000.

One unsolved problem concerning this pair of polymerizing enzymes is the relation they bear to each other. Since we are able to prepare the terminal transferase in relatively homogeneous form, we intend to use the highly purified enzyme as an antigen to see if an immunological relationship between the terminal transferase and the DNA polymerase can be detected. By this means we hope to resolve the question of whether the terminal transferase is some kind of an altered protein derived from the DNA polymerase.

18.4 POLYMERIZATION KINETICS OF THE TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE

J. M. Goncalves Patricia A. Williams
F. J. Bollum

The terminal deoxynucleotidyl transferase isolated from calf thymus gland is useful for the synthesis of a variety of homo- and copolymers. In order to understand the mechanism of polymerization and thereby to improve the yields of certain less favorable polymerizations, we have undertaken some studies on the inhibition of the enzyme reaction.

The terminal transferase is extremely susceptible to inhibition by a variety of compounds. Table 18.4.1 shows the results of some of our preliminary inhibition studies. It may be seen in category 1 that the polymerization of dATP is inhibited by the presence of any other triphosphate, but, at the same time, separate experiments show that copolymer synthesis does ensue. Pyrophosphate inhibition has been studied before, and since it is a product of the reaction, it might be expected to inhibit. We have never, however, been able to observe a reversal, that is, a pyrophosphorolysis, of the terminal transferase reaction.

In addition to the substrates that interfere with the polymerization, a variety of nonsubstrates are found to be inhibitory. Thus, referring to category 2, although ribo ATP, dADP, or any other compound in this category is absolutely not polymerized by the terminal deoxynucleotidyl transferase, each, nevertheless, shows a rather remarkable inhibition of dATP polymerization. In addition to the nucleotide derivatives, tripolyphosphate itself is a potent inhibitor, indicating that one of the important sites for enzyme action is a polyphosphate binding site.

Table 18.4.1. Inhibition of dATP[a] Polymerization

Category		Concentration (m*M*)	Percent Inhibition
1. Substrates or products	dITP	1	52
	dCTP	1	92
	dGTP	1	75
	dTTP	1	87
	PP_i	1	31
2. Nonsubstrates	ribo ATP	1	75
	dADP	2	78
	dCDP	2	78
	dGDP	2	65
	dTDP	2	75
	dAMP	1	24
	dCMP	1	12
	dGMP	1	53
	dTMP	1	31
	PPP_i	1	78
3. Sulfhydryl inhibitors	Iodoacetate	10	83
	Iodoacetamide	10	14
	N-Ethylmaleimide	10	17
	Iodosobenzoate	0.2	0
	p-Chloromercuri-benzoate	1×10^{-3}	39

[a]dATP concentration of 1 m*M*.

The sulfhydryl inhibitors, category 3, are rather interesting, particularly *p*-chloromercuribenzoate, which is effective at an extremely low level. The molecular weight calculated from *p*-chloromercuribenzoate inhibition is in agreement with the molecular weight estimated from G-200 gel filtration.

The terminal transferase is also susceptible to ionic strength inhibition and has remarkably different kinetic properties in different buffer systems. Since the kinetics of the polymerization are complex and change from buffer system to buffer system, no adequate description of the polymerization kinetics has yet been devised. This problem will be continued, and it is hoped that more quantitative kinetic constants will be derived.

18.5 FURTHER CHARACTERIZATION OF THE REACTION CATALYZED BY DNA POLYMERASE FROM *MICROCOCCUS LYSODEIKTICUS*

B. K. Zimmerman Patricia N. Howard

Studies on the DNA polymerase from *M. lysodeikticus* have established that the enzyme is qualitatively similar to other bacterial DNA polymerases. Of particular interest, however, are the vast differences observed in the priming ability of different DNA's and polynucleotides, and the divalent-ion requirements of the reaction in the presence of these various primers. One important requirement for priming activity has been shown to be the availability of terminal 3′-OH groups. Treatment of DNA with alkaline phosphatase or a DNase which increases the number of 3′-OH groups through chain breakage invariably results in a substantial increase in priming activity. However, terminal groups alone cannot account for many of the observed differences. Composition, secondary structure, and the species and concentration of divalent ion present also play an important role in the effectiveness of a given primer. Some typical results are shown in Table 18.5.1.

The products of both DNA and polynucleotide primed synthesis have been analyzed and found to reflect the overall composition of the primer. Physical characterization of the polymers synthesized is currently being carried out.

Improved methods for the isolation of DNA polymerase and other enzymes involved in DNA metabolism are being explored in detail.

Table 18.5.1. Relative Polymerization Rates with DNA Polymerase for Various Primers in the Presence of Mg^{2+} and Mn^{2+}

Primer	Optimum Mg^{2+} Concentration (*M*)	Relative Polymerization Rate	Optimum Mn^{2+} Concentration (*M*)	Relative Polymerization Rate
Native calf thymus DNA	3×10^{-3}	1.0	10^{-4}	3.6
Heated calf thymus DNA	10^{-2}	3.1	2×10^{-4}	3.3
dAT:dAT	5×10^{-3}	13	4×10^{-5}	17
Native *B. subtilis* DNA	2×10^{-3}	0.60	2×10^{-4}	0.57

Biophysics

Radiation Biophysics (19.1–19.3)		Molecular Biophysics (19.4–19.7)	
M. L. Randolph	J. L. Hosszu	R. B. Setlow[c]	W. L. Carrier
M. B. Lion[a]	R. S. Stafford	L. G. Caro	R. W. Williams
	T. P. Pitner[b]	J. E. Donnellan, Jr.	B. S. Baker[b]
		J. R. Einstein[c]	Diane M. Cottingham[b]
		R. A. McGrath	Barbara Hamkalo[e]
		J. D. Gross[a]	Maria Schnös[a]
		P. A. Swenson[d]	

[a]Visiting investigator from abroad.

[b]Student trainee.

[c]Dual assignments.

[d]Research participant.

[e]Loanee.

19.1 CHARACTERIZATION OF NATIVE AND INDUCED ELECTRON SPIN RESONANCE SIGNALS IN SEEDS

M. L. Randolph John A. Heddle[1]
A. H. Haber[2]

Introduction. – Various investigators have suggested correlations between radiobiological effects and radiation-induced free radicals. As a background for further contemplated experiments, we have used electron spin resonance (ESR) techniques to characterize free radicals in normal seeds and after several physical treatments, largely within the range of biological viability. Although logically many resonant species may be present, one tends to discuss single narrow-resonance signals as a single species because the instrumentation does not clearly resolve the species. In part, our work is intended to resolve single-line spectra into components and to differentiate between single-line spectra by application of multiple physical criteria.

Results. – In all the seed materials studied (*Vicia* root tips, New York and Grand Rapids lettuce, several types of onion, wheat, and barley) we find a 500-gauss-broad (at X band) six-peak set centered about 3400 gauss, where $g = 2.0$. In all but *Vicia* we find a singlet at ~3400 gauss. The broad set has the number of peaks and overall width of the Mn^{2+} ion, but rather than uniform-height peaks, the peak at $g \cong 2.15$ is the largest. The set of broad peaks is most prominent in *Vicia* and is unchanged by the physical treatments we have used. A narrow peak, 4 to 7 gauss wide, appears at about 3400 gauss for all but *Vicia*. However, judging from differences in line width, g value, and relaxation time as indicated by power saturation measurements, not all the central signals of different materials with different treatments are identical. In normal seeds the narrow signal is very prominent in all onion species and Grand Rapids lettuce, but small in New York lettuce and

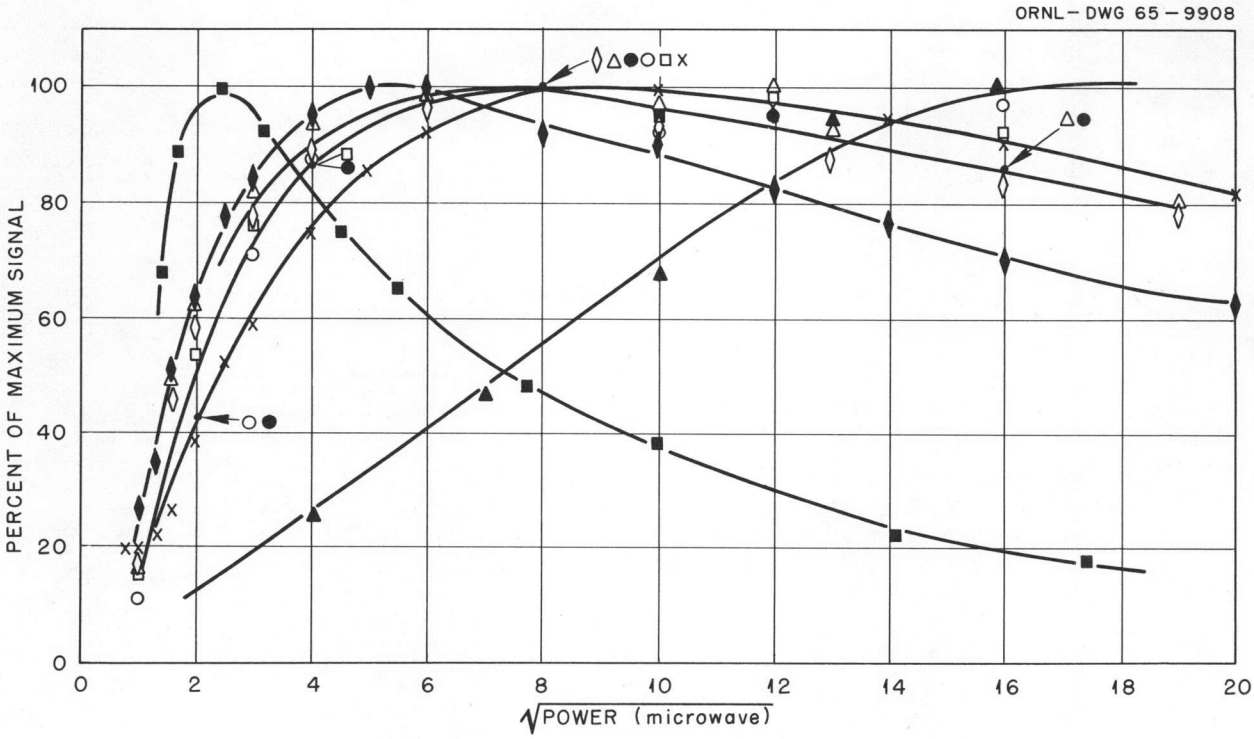

Fig. 19.1.1. Power Saturation of ESR Signals in Seeds. Derivative signal heights vs square root of microwave power. New York lettuce seed: normal △, curve 1; soaked 90 min and dried ◊, curve 1; irradiated ■, curve 2; normal $g = 2.15$ signal ▲, curve 3. Vicia: 6 kr, ○; 600 kr, ●; heated at 105°C for 2 days □; curve 4. Onion: normal ♦, curve 5; heated at 80°C for 2 days x, curve 6.

barley. Prolonged heating of dry seeds (80°C in air for two days) produces a strong added narrow signal in *Vicia* and all onions and a small signal in lettuce (New York), wheat, and barley. In *Vicia* and white globe onion, the line widths and spectroscopic g values differ marginally between the normal and heat-generated signals. Essentially negative effects for New York lettuce after short heating (100°C for 15 min), soaking, and/or boiling for 15 min followed by drying are reported elsewhere.[3] In Fig. 19.1.1 are given power saturation curves for normal and induced resonances in *Vicia* root tips, onion, and lettuce seeds. In lettuce seeds the curve for narrow resonances is unchanged by brief heat treatments, by soaking, or brief boiling, but markedly different curves obtain for the radiation-induced signal and the largest of the broad set of resonances. Heat-treated onion produces a different curve from normal onion. In *Vicia* the radiation-induced and heat-induced signals have different saturation characteristics. None of the derivative ESR curve shapes checked is broader than a Lorentzian distribution, but this finding does not exclude the possibility of overlapping slightly displaced Gaussian curves.

Conclusion. – Application of multiple physical criteria is a prerequisite to comparisons of ESR signals and may discriminate between seemingly identical signals in seeds following various treatment.

References

[1]Cytology and Genetics section.

[2]Plant Physiology and Photosynthesis section.

[3]See A. H. Haber and M. L. Randolph, "Studies of Germination and of X-Ray-Induced ESR Signals in Lettuce Seeds After Hydration and Redrying," this report.

19.2 CORRECTIONS FOR INHERENT TIMING ERRORS IN IRRADIATIONS

J. L. Hosszu M. L. Randolph

All electrically or mechanically operated switching devices involve a transient time resulting from a finite shutter speed, source or sample motion time, rise of source potential, etc. The integrated exposure after n single exposures of duration t will be

$$E = nR(t + \tau) , \quad (1)$$

where R is the steady-state exposure rate and τ the timing error. In this formulation what is probably a smooth transition from zero to full output is replaced by a step function of time. The error time given here is a serviceable idealization based on this assumption. Our technique for expeditious evaluation of τ imposes a selection rule for n and t so that

$$nt = \text{constant} = K . \quad (2)$$

Experimental values of τ are then found by fit to the equation

$$E = KR + R\tau n . \quad (3)$$

For a typical x-ray machine we find the 95% confidence ranges for R and τ to be 151.2 ± 1.3 r/min and 0.47 ± 0.03 sec. Once τ has been determined, the daily calibration value R_c is $E_c/(t_c + \tau)'$, and the proper clock setting for an exposure E' is $t' = E'/R_c - \tau$. Neglect of τ results in serious errors in E' when both (1) t' and t_c differ greatly and (2) α is comparable to either t' or τ.

19.3 ULTRAVIOLET PHOTOPRODUCTS IN 5-BROMOURACIL-SUBSTITUTED DNA OF *ESCHERICHIA COLI* 15T⁻

M. B. Lion

Introduction. – The work reported previously[1] has been continued and is summarized under the following headings: (1) 5-bromouracil (BU) photoproducts at various doses, and (2) excision of photoproducts from DNA.

Methods. – Either ^{3}HBU and ^{14}CT (thymine) or ^{14}CBU and ^{3}HTdR (tritiated thymidine) were used for preparing doubly labeled DNA. The ratio BU/(BU + T) was about 0.75 unless otherwise indicated. Other procedures were the same as before.[1]

Results. – The following results were obtained.

1. Longer chromatography times allowed detection of a fifth photoproduct from BU-DNA with $R_f = 0.36$. It is formed in about 0.5% yield at 2×10^4 ergs/mm^2 at 280 mμ. A further test, with a larger amount of material, has been made to split the isolated BU photoproducts by 254 mμ. At a surface dose of about 3×10^5 ergs/mm^2 the materials with R_f's 0.10 and 0.19 yielded up to 10% uracil (identified by its R_f). The rest of the material remained unchanged under these conditions.

The yield of the BU photoproducts at 280 mμ was measured at various doses. At the lowest dose (700 ergs/mm^2) the total photoconversion was only 0.3%, which is less than the total pyrimidine dimers produced in the DNA of unsubstituted cells. Therefore at doses in the biological range, BU-DNA irradiated in vivo seems not to be significantly more sensitive than normal DNA as measured by the respective photodecomposition of BU and T. This is in contradiction to the results obtained by K. C. Smith.[2]

2. Pyrimidine dimer excision was measured in BU-substituted DNA at substitution levels of 40 and 70%. DNA labeled with ^{14}CBU and ^{3}HTdR was used. No significant change in the rate of excision of pyrimidine dimers as compared to the rate in unsubstituted cells could be found in cells irradiated at 265 mμ with ~200 ergs/mm^2.

At zero time postirradiation incubation, the number of pyrimidine dimers formed decreased with an increase in the level of BU substitution, as expected from the assumption of random BU substitution.

The BU photoproduct of R_f 0.10 alone or together with the material at R_f 0.19 was tested for possible excision from the DNA of BU-substituted cells irradiated at 265 mμ with 200 ergs/mm^2. Separation from the other photoproducts and BU was accomplished by twp-dimensional chromatography on butanol–acetic acid–water. Results so far indicate that during 15 min of postirradiation incubation about 50% of these products disappear from the TCA-insoluble fraction. Very little disappears thereafter. The possible appearance of these products in the TCA-soluble fraction is still to be tested.

References

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[2]K. C. Smith, *Photochem. Photobiol.* 3, 1 (1964).

19.4 INTRACELLULAR REPAIR OF DNA IN X-IRRADIATED *ESCHERICHIA COLI* B/r

R. A. McGrath R. W. Williams

Introduction. – The molecular weight of DNA can be determined by measuring its rate of sedimentation in sucrose gradients. We have used this method to study DNA from *E. coli* and have measured differences in the molecular weights of single strands which result from exposure of cells to x rays. Since DNA is susceptible to breakage by shearing, we ruled out all preparative methods involving shaking, pipetting, or stirring. Cells were made into protoplasts and lysed with alkali directly on top of alkaline sucrose gradients. This procedure gives 90% recovery of high-molecular-weight denatured DNA without intervening preparative steps. It permits us to observe small numbers of x-ray-induced breaks per genome.

Results and Conclusions. – The DNA of *E. coli,* strain B/r, was labeled with ^{3}H-thymidine. Cells were exposed to x rays and incubated at 37°C for different times, then lysed and centrifuged on alkaline sucrose gradients (5 to 20%, 0.1 *M* NaCl, pH 12.0). The average molecular weight of the denatured DNA was calculated from the observed sedimentation values. Results shown in Table 19.4.1 indicate that (1) DNA assayed by these methods has an average molecular weight of 1.58×10^8 daltons [approximately one-eighth of a single strand of the *E. coli* genome (molecular weight 14×10^8 daltons)], (2) molecular weight is reduced by exposure of cells to x rays, and (3) with incubation after irradiation the molecular weight increases.

We found in *E. coli* B_{s-1}, a radiation-sensitive mutant, that the molecular weight of the DNA does not increase during similar incubation after x-ray exposure even though the initial reduction in molecular weight is identical to that found in B/r. From these data we calculate that 2.5 kr (an exposure that gives 50% survival of B_{s-1}) results in one break in each strand of the intracellular DNA. We interpret these results as evidence for repair of irradiated DNA in the case of B/r by some mechanism involving restitution of broken pieces and as evidence that in B_{s-1} such a mechanism either does not exist or functions much more slowly.

Table 19.4.1. Average Molecular Weight, in 10^8 Daltons, of Denatured DNA of *E. coli* B/r

Each number represents an average value from three experimental trials.

X-Ray Exposure (kr)	Minutes of Incubation Following Irradiation		
	0	20	40
0	1.58		
10	1.09	1.10	1.71
20	0.85	1.47	1.41
40	0.56	Less than 0.9 at 60 min	

19.5 EFFECTS OF ULTRAVIOLET IRRADIATION ON *BACILLUS MEGATERIUM* VEGETATIVE CELLS

J. E. Donnellan, Jr. R. S. Stafford
R. B. Setlow

Introduction. – Bacterial spores are some six to ten times more resistant to ultraviolet (uv) irradiation than most vegetative cells. Studies in this laboratory have shown that resistance may, in part, be due to the failure of uv radiation to produce thymine dimers in spore DNA irradiated in vivo.[1] For comparisons of this type to be meaningful, the effects of irradiation on spores and vegetative cells should be compared and the effects on the vegetative cells compared to the effects on vegetative cells of non-spore-forming organisms such as cells of *E. coli*. This report concerns a study on the effects of ultraviolet irradiation of vegetative cells of *B. megaterium*.

Results and Discussion. – Labeled cells of *B. megaterium* were produced by inoculating a yeast extract medium containing ^{3}H-thymidine with a suspension of heat-shocked spores (60°C, 15 min).

After 2 hr, the cells were well into exponential growth and were then diluted with buffer for irradiation. The fraction of thymine dimers in irradiated cells was determined by acid hydrolysis of cells followed by isolation of dimers and thymine by paper chromatography and counting in a scintillation counter. A dose of 600 ergs/mm^2 at 2650 A resulted in 0.37% of the thymine appearing in dimers ($\widehat{UT}$ + $\widehat{TT}$) and a survival of 5%. Incubation of the irradiated samples under yellow lights for 30 min resulted in the disappearance of 65% of the thymine dimers from the acid-insoluble fraction and their appearance in the acid-soluble portion of the sample. Photoreactivation with 4050-A radiation produced almost complete recovery of colony-forming ability and a disappearance of thymine dimers.

Conclusions. – Vegetative cells of *B. megaterium* react to ultraviolet irradiation in the same manner as the vegetative organism *E. coli* – first, in the production of thymine dimers in their DNA; second, by removal of these lesions by excision during dark repair or by "splitting" during photoreactivation. In spores of this organism, on the other hand, thymine dimers are not produced by uv, but another photoproduct is found. However, this photoproduct is also removed from the acid-insoluble fraction by a dark repair mechanism. The repair mechanisms of spores and their vegetative cells will be compared in order to better understand the mechanism of cellular repair from radiation damage.

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19.6 THE v GENE AND THE EXCISION OF DIMERS FROM BACTERIOPHAGE DNA

R. B. Setlow W. L. Carrier

Bacterial strains that are resistant to the effects of uv radiation possess enzymic systems that can repair damaged DNA. The general steps in the repair process seem to be the excision of uv-induced lesions (primarily pyrimidine dimers) from the bacterial DNA followed by resynthesis of DNA to fill in the excised region. Sensitive strains are deficient in one or more of these steps.[1] Bacteriophage mutants are known whose uv sensitivity is under genetic control. For example, T4 phage is twice as resistant as T2 phage, and this difference is associated with the presence of the v gene in T4 – T4 is v$^+$ and T2 is v$^-$. Harm[2,3] has shown that v$^+$ phages carry information that enables an infected bacterium to repair uv damage in phage DNA in the dark and that this reparable damage is similar to that repaired by photoreactivation. Since photoreactivation involves monomerization of pyrimidine dimers,[4] one would naturally predict that the v gene acts by an excision mechanism similar to that found in bacteria. We have looked for and found such an excision mechanism.

Methods. – Phages T4v$^+$ and T4v$^-$ were labeled with ^{3}H-thymidine. They were irradiated with doses of 200 ergs/mm^2 at 265 mμ (approximately five lethal hits to T4v$^+$), and as a result, 0.15% of their thymine was in thymine dimers. These phages were used to infect *E. coli* B or *E. coli* B_{s-1} (a uv-sensitive strain) at a multiplicity of infection of 5. The infected cells were disrupted by sonication at various times during the latent period, and the cellular contents were fractionated on alkaline Sephadex columns into high-molecular-weight (DNA) and low-molecular-weight (oligonucleotides) components. Each component was analyzed for thymine dimers.[1]

Results. – The data for a number of different DNA's are shown in Table 19.6.1. Data not given in Table 19.6.1 indicate that any dimers not found in the DNA fraction were observed in the oligonucleotide components. The results are clear. (1)

Table 19.6.1. Radioactivity in Dimers Relative to Total Thymine, $\widehat{TT}$/T, in Phage DNA Before Infection and 20 min After Infection

Ultraviolet-Irradiated Phage	Bacteria	$\widehat{TT}$/T in Percent	
		Zero Time	After 20 min
T4v$^+$	B	0.15	0.02
	B_{s-1}	0.15	0.01
T4v$^-$	B	0.15	0.12
	B_{s-1}	0.16	0.14
T4v$^-$ plus cold T4v$^+$	B_{s-1}	0.15	0.06

The presence of the v gene results in rapid excision of dimers from the phage DNA, whereas in its absence there is negligible excision. (2) A mixed infection with v^+ and v^- phages results in the excision of dimers from the DNA of the v^- phage. (3) The enzyme(s) in *E. coli* B that excise dimers from bacterial DNA do not excise dimers from phage DNA. This result explains why the survival of irradiated T-even phage is independent of the strain of titering bacteria.

References

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19.7 DNA TRANSFER IN BACTERIAL CONJUGATION

L. G. Caro J. D. Gross[1]

Introduction. – Since the bacterial chromosome seems to be constantly undergoing replication and since replication is, like chromosome transfer in bacterial mating, a linear, orderly process, it is tempting to speculate that a relation exists between the two processes. By labeling Hfr DNA during or before mating, while the F^- was prevented from DNA synthesis by adenine starvation, we found evidence for a replication of DNA being associated with transfer so that a newly made copy is always transferred from the Hfr to the F^-.

Results. – These results could have been explained by a residual DNA synthesis in the F^-, using adenine from the Hfr. The same results were obtained, however, in experiments in which the recipient cells were prevented from synthesizing DNA during mating by prior irradiation with a heavy dose of ultraviolet light rather than by adenine starvation. These results are what would be expected if replication and transfer occurred simultaneously. There is, however, another conceivable explanation, that is, that the Hfr chromosome is transferred without any replication and is immediately replicated in the F^-. This explanation is not very plausible since one would have to assume that, when the F^- cells are starved of adenine, they either receive just sufficient adenine from the male cells to allow replication of an amount of DNA equivalent to the incoming Hfr DNA or receive more but only synthesize *that* amount of DNA. In order to eliminate this explanation we have performed an experiment which enables us to demonstrate that not more than one strand of preformed Hfr DNA is transferred during conjugation.

We compared the label transferred during conjugation by two parallel Hfr cultures, one labeled until the time of mixing with the F^-, the other placed in cold medium 1.3 generations before mixing to ensure that all the DNA molecules would be either hybrids with one hot and one cold strand or completely cold. It was found that, in the first mating, twice as many F^- cells received label as in the second but that the amount received by individual cells was the same in both cases. This experiment shows that, at most, one strand of preformed DNA can be transferred in mating.

Conclusion. – Transfer of DNA from an Hfr cell to an F^- cell seems to take place through a new round of replication initiated after contact and proceeding at the same rate as transfer so that only one of the two newly made copies is transferred. These characteristics fit the model proposed by Jacob and Brenner.[2]

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Enzymology

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Protein Synthesis (20.1–20.7)

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Enzymatic Mechanisms in Protein Synthesis (20.8–20.10)

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Mammalian Cell Regulation (20.11–20.12)

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Participation in Cooperative Projects:

NIH-AEC – Carcinogenesis Programs (24.5–24.7)

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20.1 PROTEIN SYNTHESIS BY LIVER PREPARATIONS FROM PUROMYCIN-TREATED RATS

Marialuisa Melli
Peter Pfuderer
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G. D. Novelli

Introduction. – In connection with studies of RNA synthesis in the livers of rats in which protein synthesis is inhibited by hourly injections of puromycin,[1] we observed a rather marked shift in the ribosomal profile of puromycin-treated animals. It became of interest, therefore, to compare protein synthesis both in vivo and in vitro with the ribosomal profiles of animals treated with puromycin and those recovering from the effects of the drug.

Results and Discussion. – In vivo protein synthesis (measured as ^{14}C-leucine incorporation into hot–trichloroacetic acid–precipitable material) in rats treated with puromycin for 3–4 hr starts to recover in 4–5 hr after the last injection of the drug. The recovery increases logarithmically, becoming normal at approximately 8 hr after the last injection of puromycin.

Examination of the ribosomal population in a linear 10–34% sucrose density gradient centrifuged for 2 hr at 25,000 rpm revealed that as early as

1 hr after the first puromycin injection there is a marked change in the ribosomal profile. The polyribosomes disappear almost completely, and the optical density at 260 mμ is accumulated in a broad peak sedimenting in the region between dimers and monomers, with a sedimentation rate of approximately 105S. This picture remains essentially constant throughout the entire period of inhibition of protein synthesis. If the animals are allowed to recover from puromycin inhibition and in vivo protein synthesis is measured as above, we find that, when protein synthesis returns to normal, the ribosomal population shows a sedimentation profile similar to the ones obtained from animals under puromycin inhibition.

During the following hours, polysomes start to increase in number and the distribution profile shifts towards normal.

We measured, in the analytical ultracentrifuge, the sedimentation constant of the ribonucleoprotein particles present in the postmitochondrial supernatant (PMS) of livers of puromycin-treated animals and observed several distinct peaks with sedimentation rates of 72, 61, 53, 44, and 30S. These particles are not present in control PMS measured at the same dilution. The normal PMS seems to be composed mainly of polysomes and a few monoribosomes. It is surprising that the results obtained with the analytical ultracentrifuge are so different from those obtained with sucrose density analysis. In the analytical ultracentrifuge the ribonucleoprotein particles from puromycin-treated animals exhibit many peaks with sedimentation rates much lower than are obtained in a sucrose density gradient. It seemed possible that in the sucrose density gradient the ribonucleoprotein particles aggregate to give a single heavy peak. This was confirmed when we observed that, after centrifuging the density gradients for 5 hr instead of the usual 2 hr, the patterns obtained were comparable to the ones seen in the ultracentrifuge. The area occupied by particles lighter than monoribosomes represents 80–90% of the total population.

The in vitro amino-acid-incorporating activity of the ribonucleoprotein particles from livers of rats treated for varying times with puromycin was measured. Microsomes and ribosomes were prepared from livers of rats that had been treated for 1, 2, and 4 hr with puromycin. An additional group of rats were treated with puromycin for 4 hr and then allowed to recover for 3 hr (these are called 7-hr rats).

Incorporation of ^{14}C-phenylalanine by microsomes from 1-hr-treated rats was inhibited 12% and up to 18–20% for microsomes from rats that had been treated 4 hr. Microsomes from rats treated for 4 hr and allowed to recover for 3 hr incorporated 58% more than microsomes from control rats. The ribosomes from such treated animals, on the other hand, showed a 12% inhibition of their incorporating ability.

We will continue to study in vitro amino acid incorporation by such preparations and to look also at stimulation by polyuridylic acid.

References

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20.2 POSSIBLE RECYCLING OF RIBOSOMES IN AN IN VITRO INCORPORATING SYSTEM

Lawrence Rosen G. D. Novelli

Introduction. – A cell-free system which is capable of synthesizing polypeptides has as a necessary component the ribosomal fraction. The techniques that lead to ribosomal preparations have varying degrees of activity, none of which can compare to the efficiency of in vivo protein synthesis. Thus the problem of isolating more active ribosomal preparations is of primary importance.

For a cell-free system derived from *E. coli* which incorporates amino acids into polypeptides, the incorporation is dependent on a supernatant fraction and added cofactors in addition to the ribosomal fraction. When the first two groups are kept constant, the amount of incorporation is proportional to the ribosome concentration, the latter group being composed of both active and inactive material. Ribosomes may be inactive due either to a lack of endogenous template or to denaturation, etc.

In order to determine the active and inactive ribosomal components within a given population, advantage was taken of the ability of polyuridylic acid (poly U) to stimulate phenylalanine in a cell-free system derived from *E. coli*.

Results and Discussion. – Optimal conditions were first obtained for the incorporation of phenylalanine in the presence and absence of poly U.

Under these conditions and in the absence of poly U, incorporation of phenylalanine was essentially complete in 30 min (endogenous incorporation). The addition of poly U at zero time to such a system led to an incorporation of phenylalanine which was 30 to 50% greater (increase I) than the endogenous incorporation.

When incorporation was allowed to proceed for 40 min before the addition of poly U, the amount of phenylalanine incorporation stimulated by this late addition was 150–300% greater (increase II) than the endogenous incorporation.

Increase I may be considered as mainly due to the interaction of poly U with ribosomes that were inactive due to lack of endogenous template. Increase II is probably an additive effect, one part of which is due to increase I; an additional stimulation is due to interaction of poly U with ribosomes that were released from their endogenous template during the 40-min incubation without poly U.

These results indicate that ribosomes are capable of synthesizing polypeptides in a cyclical fashion; that is, the release of active ribosomes from template RNA after polypeptide synthesis allows them to interact with added template RNA and to synthesize additional polypeptide. To test this hypothesis, an amino-acid-incorporating system was incubated for 40 min to complete endogenous incorporation. At this time, poly U was added, and 30 min later when the poly U stimulation of phenylalanine incorporation was complete, additional poly U was added. Thirty minutes later, a third addition of poly U was made. Each addition of poly U led to a further increase in the incorporation of phenyalanine, with no loss of the system's ability to respond to poly U.

Although these results are of a preliminary nature, they indicate the direction of further experiments to obtain an active class of ribosomes capable of responding to added template RNA. Thus, techniques are being explored which can lead to the isolation of large-scale quantities of ribosomes or polyribosomes with high endogenous ability to incorporate amino acids. These ribosomes may be released from their endogenous template by incubation, and the free monomers isolated. These free monomers can then be assayed by their response to added template RNA including, hopefully, natural messenger RNA for the synthesis of specific protein.

20.3 INTERACTION OF POLYURIDYLIC ACID AND RIBOSOMES

Lawrence Rosen D. C. Levin[1]
G. D. Novelli

Introduction. – The interaction of polyuridylic acid (poly U) with ribosomes has been investigated in many laboratories.[2–4] The formation of such a complex is dependent on conditions of ionic strength, Mg concentration, etc.

In the present work it is assumed that, if the ribosome/poly U ratio is large, interaction should lead to a mixed polysome population capable of being separated in a sucrose density gradient (SDG) from the noninteracting ribosomes. The resultant polysomes can then be assayed for phenylalanine incorporation by the addition of a high-speed supernatant fraction and necessary cofactors. Poly U then can be added again to the noninteracting monomer peak and the separation repeated; this procedure may be repeated until no further interaction occurs.

The use of such a technique may then be an effective assay for determining the active ribosomes in a mixed monomeric peak and for the separation of active and inactive monomers.

Results and Discussion. – An excess number of trout liver ribosomes composed of more than 95% monomers (nominally 80S) were mixed with ^{14}C–poly U at different Mg concentrations and then sedimented through an SDG. The amount of poly U which became attached to ribosomes was determined by assaying the trichloroacetic acid–insoluble ^{14}C in an aliquot from each fraction of the SDG.

The results indicated that less than 20% of the poly U became attached to the ribosomes. Thus, for a mixed monomer population, these preliminary results indicate that the greater number of ribosomes are incapable of interacting with added poly U.

Further experiments will be done to determine what proportion of the monomers do not interact due to the presence of endogenous template RNA or due to loss of competence (denaturation, etc.).

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20.4 PROTEIN BIOSYNTHESIS IN *CLOSTRIDIUM PASTEURIANUM*

R. D. MacElroy G. D. Novelli

Introduction. – Studies on protein biosynthesis in *Clostridium pasteurianum* are continuing.[1–3] Recent work has been concerned with the relationship among ribosomes, polysomes, and ribosome subunits during various phases of the growth cycle of the organism. Several methods of breaking cells have been used. In general, high pressure, sonication, and grinding have proved effective; however, all these treatments apparently cause breakdown of polysomes. It has recently been possible to rapidly break the cells by first forming spheroplasts with lysozyme and then lysing the cells with osmotic shock or detergent. Under these conditions, polysomes are preserved, and it is possible to resolve polysomes containing up to eight ribosomes in sucrose density gradients.

Results and Discussion. – Lysis of spheroplasts by osmotic shock or by a nonionic detergent such as Brij-35 releases about 60% of the total cellular RNA. After removing the cell debris, sucrose density gradients of the supernatant indicate that the largest fraction of the RNA is in ribosomes and polysomes. Polysomes constitute about 40% of the total ribosome material. There is also a significant fraction of ribonucleoprotein which sediments in a region lighter than the monosome, as well as a peak containing soluble protein and transfer RNA.

When the spheroplasts are lysed with deoxycholate (DOC), about 80% of the RNA is released, and the ratio of polysomes to monosomes increases slightly when compared to Brij-lysed spheroplasts; the most noticeable difference, however, is the appearance of a large peak in the region between the monosome peak and the peak of the transfer RNA. When the sedimentation constant of the monosome is assumed to be 70S, the sedimentation constant of the DOC released peak is about 45S.

If the spheroplasts are first lysed with Brij-35 and the sedimented cell debris is then extracted with DOC, polysomes are released and constitute about 80% of the released ribosomes. More striking is the appearance of two peaks with sedimentation constants of roughly 36 and 53S. These peaks contain about 50% of the ribonucleoprotein released from the debris. The addition of DOC to Brij-extracted material has no effect on the pattern of the gradient.

Whole cells incorporate labeled orotic acid into material which sediments in sucrose density gradients and corresponds to the optical density peaks found in the DOC extract of the debris. With fairly long pulses, the specific activity of the 36S peak is greater in the extract from the Brij-lysed cells than in DOC supernatant.

We are presently attempting to detect newly synthesized RNA which sediments in the region between the monosome and the transfer RNA and to determine the significance of the material which is released from the cell debris by DOC.

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20.5 STUDIES ON POLYSOMES IN RAT SPLEEN DURING THE IMMUNE RESPONSE

C. J. Wust Johanna C. Parkins

Introduction. – In a continuing study to understand the events of the induction phase of the immune response, attention has been directed to the ribosomal population of rat spleen homogenates following immunization with sheep erythrocytes.

Results and Discussion. – Microsomes and microsomes treated with sodium deoxycholate have been prepared from homogenates of rat spleen. The procedure involves homogenization of the rat spleens in a medium containing: 0.01 *M* Tris acetate, pH 8.0; 0.01 *M* magnesium acetate; 0.1 *M* KCl; 0.25 *M* sucrose; and 5.5 mg/ml of washed bentonite. After centrifugation at 10,000 and

20,000 × *g*, the supernatant fluids were diluted with 1.5 volumes of a medium similar to the above except that it contained 0.9 *M* sucrose. The mixture was centrifuged at 32,000 × *g*, and the supernatant was centrifuged directly at 105,000 × *g* to obtain microsomes or treated with 0.65% sodium deoxycholate before centrifugation at 105,000 × *g* to obtain ribosomes. The particles thus obtained were sedimented on a 0.25 to 1 *M* sucrose density gradient at 25,000 rpm for 120 min, and sedimentation coefficients were determined in a Spinco model E ultracentrifuge. Under these conditions, six peaks of optical density at 260 mμ were observed representing 65, 81, 105, 120, 155, and 174S. The predominant peak was the 120S (dimer). Although material having optical density at 260 mμ was seen sedimenting at a density greater than 174S, it could not be resolved with the use of exponential gradients. Identical optical density profiles were found with both microsomes and deoxycholate-treated microsomal preparations. Microsomal preparations were more turbid than deoxycholate-treated preparations, but could be clarified by centrifugation at 5000 × *g* before being subjected to density-gradient centrifugation. There were no differences in the optical density profiles of microsomes obtained from the spleens of rats immunized to sheep erythrocytes. The spleens were removed and examined at 24, 48, 72, 96, 168, and 216 hr after injection of antigen.

These ribosomal preparations were stored in liquid nitrogen, and their capacity for amino acid incorporation in a cell-free system determined as a function of time after storage. In contrast to our previous findings that incorporating ability was rapidly lost after 5 days of storage,[1] these preparations were fully active after 30 days. The relative amount of ^{14}C-labeled leucine incorporated per milligram of RNA was not significantly different during immunization, with the exception of particles obtained at 72 hr. At 72 hr, which is the time of logarithmic increase in concentration of circulating hemagglutinin, the preparations were found to have a twofold increase in incorporation over particles prepared from the spleens of nonimmunized rats.

After 30 min of incorporation, the incubation mixture was subjected to centrifugation over 0.25 to 1 *M* sucrose density gradients. The characteristic feature of the findings was that there was a loss of the 120S (dimer) and 81S (monomer) optical density peaks and an increase in the 65 and 105S peaks. Hot-acid-insoluble material was found throughout the gradient tube with an almost constant specific activity.

In vivo labeling of ribosomal RNA with ^{32}P-orthophosphate has confirmed previous findings.[2] A 2-hr pulse with ^{32}P labeled all ribosomal classes. With so-called microsomal preparations, there was material sedimenting toward the bottom of the tube with a very high specific activity. This material was observed with microsomes obtained from spleens of nonimmunized rats and spleens of rats immunized for 24 hr. In the latter case the specific activity was two to three times greater than the former. This material of high specific activity was not seen in spleens of rats immunized for periods longer than 24 hr. The labeled material was lost by treatment with deoxycholate and indicated the only difference found between "microsomal" and "deoxycholate-treated microsomal" preparations. The relative specific activity of the ^{32}P label increased, with immunization being about fourfold at 72 hr over preparations from nonimmunized animals. It then decreased and was not significantly greater than preparations from nonimmunized rats at 6 to 9 days after injection of antigen.

References

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20.6 CONFIGURATION OF ISOLATED AND EMBEDDED POLYSOMES

Peter Pfuderer D. C. Swartzendruber[1]

Deoxycholate-treated liver polysomes were prepared from rat liver, pelleted, fixed in glutaraldehyde, embedded in Epon, and observed by thin-section electron microscopy.

Sections of C-ribosome pellets, containing roughly 15% by weight of polysomes that were nine ribosomes or larger, disclosed very few helical polysomes. Partial fractionation of these C-ribosomes by centrifugation yielded a pellet containing 30% polysomes of nine units and larger. Sections from this pellet yielded of the order of two ribosomes in a helical polysome array per

Fig. 20.6.1. Thin-Section Electron Micrograph of Liver Polysomes. Helical structures are indicated by arrows.

hundred ribosomes. By further fractionating the polysomes in a zonal ultracentrifuge, a pellet was obtained which was composed of polysomes ten units long or larger. Micrographs from this pellet disclosed from two to five ribosomes in a helical array per hundred ribosomes. Figure 20.6.1 shows a micrograph of a section of this pellet. Randomly oriented helical polysome rods in a section 600 to 900 A thick could have a maximum angular deviation of the order of 40° from the plane of the section and still contain three turns of the helix in the section and be recognizable as a helix. The probability of the rod being correctly oriented would then be of the order of 0.1 = 40/360. The probability of finding a helical polysome in a pellet would then be 0.1 times the fraction of the polysomes large enough to form a recognizable helix. The three pellets described should then have shown 1, 2, and 10% helical polysomes if all the polysomes were helical, while the number found was roughly 0.1, 2, and 3% respectively. It can be concluded that of the order of 10% or more of polysomes of nine ribosomes long or larger are in a helical array in the pellets examined.

References

[1]Mammalian Recovery section.

20.7 MONOMER-POLYMER EQUILIBRIA IN ADENOSINE-5′-PHOSPHOSULFATE REDUCTASE

Peter Pfuderer H. D. Peck, Jr.
D. R. Holladay

Introduction. – The enzyme adenosine-5′-phosphosulfate reductase (APS reductase) has recently been obtained in a state of high purity by Peck, Deacon, and Davidson.[1] This study describes some physical properties of this enzyme preparation.

Exposure of the enzyme to 0.01 *M* sodium dodecyl sulfate results in a lowering of the apparent

molecular weight of the enzyme from 150,000 to values of 20,000 or so.[2] Similarly, iron analyses of the enzyme indicate from five to six irons per flavin adenine dinucleotide molecule, giving 24,000 to 30,000 as the value of the smallest iron-containing unit. This data was indicative of the existence of subunits.

The sedimentation rate of the enzyme was found to increase from 10.5 to 12.5S as the temperature was lowered from 30 to 0°C. Over the same temperature range the intrinsic viscosity was virtually unchanged. Therefore, the species existing at 25°C was capable of further aggregation in the cold, as the following table indicates.

Temperature (°C)	S (svedbergs)	Molecular Weight (viscosity, sedimentation)
25	10.5	228,000
10	10.8	238,000
5	11.3	255,000
1	12.2	286,000

A Sephadex G-200 column separation of the native enzyme followed by concentration and centrifugation of the isolated fractions showed the existence of polymers of APS having sedimentation rates of 2.7, 6.9, 10.6, 13.6, and 27S corrected to water at 20°C. A molecular weight determination of the partially purified 2.7S species led to a value of 37,000 for this species. A homogeneous species of 140,000 molecular weight has also been isolated which must correspond to the 6.9S polymer. It seems likely that these sedimenting species are all aggregates of the 2.7 monomer, the 2.7, 6.9, 10.5, and 13.6S species apparently being composed of one, four, six, and eight subunits respectively.

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20.8 ISOLATION AND PURIFICATION OF AMINO-ACID-ACTIVATING ENZYMES

M. P. Stulberg K. R. Isham
Jane B. Mills

Introduction. – Our efforts to purify the phenylalanine-activating enzyme (Phe AE) from *E. coli* have resulted in the production of a highly purified fraction.

Results and Discussion. – Originally we isolated Phe AE from a by-product of the purification of another enzyme. However, we discovered that the by-product contained only a fraction of the Phe AE activity when compared to the amount present in a cell extract. Therefore we have now begun isolating the enzyme directly from cells and have altered our procedure somewhat. The purification protocol consists of cell breakage, centrifugation to remove particulate matter, streptomycin precipitation to remove nucleic acids, ammonium sulfate fractionation, and DEAE and hydroxylapatite column chromatography. This procedure has produced a 1000-fold-purified enzyme with a 50% yield. The original procedure was developed by starting with 100 g of cells and has been scaled up to work starting with 500 g of cells. Further increases in capacity can be easily accomplished because of the simplicity of the procedure.

We are presently testing the purified enzyme preparation for homogeneity and the presence of other activating enzymes, and we are establishing the kinetics and other properties for use in future experiments. Experiments to elucidate the mechanism of the interaction of the enzyme with its specific transfer RNA[1] are also now in progress.

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20.9 ISOLATION AND PURIFICATION OF SPECIFIC TRANSFER RNA FROM *E. COLI*

A. D. Kelmers[1] G. D. Novelli
M. P. Stulberg

Introduction. – We have now accomplished essentially a complete separation of phenylalanine

transfer RNA (Phe tRNA) from the other tRNA's of *E. coli*. Progress has also been made in the separation and isolation of the other tRNA's.

Discussion and Results. – Phe tRNA has been obtained free of other tRNA's, with only a contamination of inactive RNA remaining. This progress has been made possible only after testing the effects of pH, temperature, Mg^{2+} concentration, etc., on the elution pattern obtained from the reverse-phase-chromatography columns. In order to obtain maximum purification and yield of Phe tRNA, the columns are operated at pH 4.5 at 37°C and with a 0.1 *M* Mg^{2+} concentration. Ranges in pH from 4.5 to 7.0 and in temperature from 10 to 45°C have demonstrated that the use of specific conditions will allow the separation of many of the remaining tRNA's.

Our starting preparations of crude tRNA have been improved by the use of molecular sieving columns that remove approximately 35% inactive RNA.

We have initiated the use of hydroxylapatite columns as another means of separating tRNA's. Preliminary results indicate good resolution and separation, and the method will probably be used to supplement the separations obtained with the reverse-phase-chromatography columns.

References

[1]Chemical Technology Division.

20.10 EFFECTS OF METHYLATION ON PHENYLALANINE TRANSFER RNA FROM *E. COLI*

L. R. Shugart M. P. Stulberg

Introduction. – The question of the role of methylated bases in tRNA has been examined quite thoroughly by several laboratories, but no significant answers have been obtained. In all these investigations a mixture of tRNA's as well as crude enzymes was used for testing, thus making any results obtained difficult to interpret. We shall attempt to isolate Phe tRNA's[1] containing varying amounts of methylated bases and test their activity with the use of the purified phenylalanine-activating enzyme from *E. coli*.[2] It is felt that when the effect of methylation is tested with purified components, a clear-cut answer will be forthcoming as to whether methylation of tRNA affects the biological activity of these nucleic acids.

Results and Discussion. – Initial experiments have been directed toward a study of the growth conditions of *E. coli* K12-W6 (402) that result in the maximum synthesis of undermethylated tRNA during starvation for methionine. The "relaxed" mutant supposedly will double its content of RNA without concomitant growth. Because the mutant requires methionine for growth and since methionine is the source of methyl groups for tRNA, we plan to isolate the undermethylated Phe tRNA after a period of "relaxation."

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20.11 ISOZYMES: THEIR REGULATION AND GENETIC CONTROL

E. H. Grell[1] J. B. Murphy
K. B. Jacobson Eunice L. Kincaid

Introduction. – We have been studying various aspects of the existence of multiple forms of an enzyme (isozymes). The reason for the existence of multiple forms of an enzyme presumably is for the regulation of metabolic reactions. The regulation of the isozymes themselves occurs at at least two levels. In the case of the lactate dehydrogenases of higher animals the distribution among the multiple forms is controlled by the organ, whereas the type of protein subunits available to the organ is controlled genetically (presumably). Neither the mechanism by which the organ determines the distribution of lactate dehydrogenase isozymes nor complete genetic evidence for the control of the various subunits has come forth. Our attention has turned to a system of isozymes for alcohol dehydrogenase found in *Drosophila melanogaster*.[2] The genetic control of the isozyme system has been shown to reside at a single locus of chromosome 2, and yet there are clearly at

least three electrophoretic forms of alcohol dehydrogenase in each of the homozygotes. The enzyme pattern of the hybrid is consistent with a dimer form of the enzyme molecule.

Enzyme Purification. – One hypothesis to explain the isozyme pattern of alcohol dehydrogenase involves a conversion of one isozyme to another. To explore this, the alcohol dehydrogenase is being purified. The enzyme is obtained at least 50% pure on a protein basis after being subjected to ammonium sulfate fractionation, filtration through DEAE-cellulose and cellulose phosphate, and chromatography on hydroxylapatite. The separation of the electrophoretic forms, however, has not been achieved. Further attempts at fractionation are being made. Characterization of the two electrophoretic forms reveals rather marked differences.

Search for Mutants. – Another hypothesis suggests that the genetic locus found for the alcohol dehydrogenase is a regulator gene and that the genes specifying the protein structure are different and separate. Mutant forms of the enzyme would provide the opportunity to test this hypothesis. Ethylmethane sulfonate was injected into adult *Drosophila*, and the progeny were examined for abnormal forms of the isozymes. Several such aberrations were found, but none bred true, suggesting that they were mosaics. The search for stable mutants is continuing.

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20.12 CODING PROPERTIES OF tRNA

K. B. Jacobson W. E. Barnett[1]

Previously we described an aminoacyl tRNA synthetase in *Neurospora crassa* that attaches phenylalanine to the tRNA's of *E. coli* that are specific for alanine and valine. The position of this phenylalanine when incorporated into a peptide can be a test of the mechanism which regulates the sequence of amino acids in protein synthesis.

^{14}C-Phe $tRNA^{ala,val}_{coli}$ was prepared by the *Neurospora* enzyme and added to the ribosomes from rabbit reticulocytes. The phenylalanine was transferred readily from the tRNA into peptide material. The hemoglobin produced by the ribosomes was converted to globin, and the globin was isolated chromatographically and digested with trypsin. The resulting peptides were fractionated on a Dowex 1 formate column, and the elution pattern compared with that of ^{3}H-phenylalanine–labeled hemoglobin (as produced by intact reticulocytes). The patterns were dissimilar.

Although the positions of the ^{14}C-phenylalanine have not yet been shown to be identical with those of alanine and valine in the hemoglobin, this result may be inferred and direct evidence obtained to confirm it.

References

[1]Nucleic Acid Enzymology section.

Biochemical Regulation

F. T. Kenney[a] W. D. Wicks D. D. Holten[b]	Joseph Kendrick L. E. Roberson[a] W. L. Albritton[c] Anne S. Faust[d]	*Participation in Cooperative Projects* NIH-AEC – Carcinogenesis Programs (24.4)

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21.1 STIMULATION OF TRANSFER RNA SYNTHESIS BY HYDROCORTISONE

W. D. Wicks F. T. Kenney

Introduction. – Our continuing studies[1,2] on the general stimulation of RNA synthesis by steroid hormones have recently been directed at determining whether there is any selectivity in the observed response in rat liver after the injection of hydrocortisone. As a test of this possibility we have used a double-isotope procedure coupled with the technique of Kelmers *et al.*[3] for the separation of transfer RNA (tRNA) into its component species.

Results and Discussion. – ^{14}C-Orotic acid was injected intraperitoneally into control rats and ^{3}H-orotic acid into rats given hydrocortisone simultaneously. One hour later another injection of the two isotopic precursors was given, and after 60 min the rats were killed. Livers from both groups were combined and homogenized in, and shaken with, 0.14 *M* NaCl and phenol at 3°. Transfer RNA was separated from the phenol-extracted RNA by treatment with 1 *M* NaCl. The isolated tRNA was incubated at pH 10.3 at 37° for 90 min to remove bound amino acids and was reprecipitated. The resulting RNA was then applied to a 150- by 1-cm column of dimethyldilaurylammonium chloride dissolved in isoamyl acetate fixed on Chromasorb G and chromatographed with a linear gradient of 0.4–0.7 *M* NaCl with or without a gradient of $MgCl_2$ superimposed. Approximately 150 fractions of 10 ml each were collected.

Although the pattern varied somewhat, there was considerable spreading of the tRNA, indicative of a significant degree of separation. Aliquots of each tube were counted, and the ratio of ^{3}H to ^{14}C in each sample was determined. In three separate experiments no major shift in the ratio of ^{3}H to ^{14}C was observed in any fraction. Reversing the isotopes reversed the value of the ^{3}H to ^{14}C ratio but did not alter its relative constancy. Within the limitations of the techniques employed, we have concluded that the degree of selectivity in the effect of hydrocortisone on tRNA synthesis is probably low. It is possible, however, that more refined techniques may yet uncover a degree of specificity heretofore undetected.

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21.2 RNA SYNTHESIS IN FETAL RAT LIVER

W. D. Wicks F. T. Kenney

Introduction. – It has been known for several years that the level of tyrosine transaminase in rat liver rises dramatically soon after birth, reaching the adult level by 12 hr or so. Although this postnatal increase can be largely prevented by adrenalectomizing the fetus, the administration of hydrocortisone to the fetus is incapable of elevating the very low level of enzyme activity in the liver prior to birth. As an adjunct to our studies on the effects of hydrocortisone on enzyme induction and RNA synthesis, we have initiated studies on the effects of gestation and birth on the synthesis of RNA in rat liver. We hope to correlate changes in enzyme levels with changes in the pattern of RNA synthesis. Attempts will be made to elucidate the mechanisms underlying the sudden shift in tyrosine transaminase activity at birth and the role which hydrocortisone plays in this process.

Table 21.2.1. Composition of Bulk and Pulse-Labeled RNA from Adult and Fetal Rat Liver

	Adult	Fetal
Mole Percent		
AMP	19	19
GMP	34	31
UMP	20	20
CMP	27	30
Percent of Total $^{32}P^{a}$		
AMP	25	20
GMP	25	25
UMP	25	26
CMP	25	29

[a]After 20 min labeling period.

Preliminary experiments have shown that the fetal liver is very active in synthesizing RNA just before birth. Sucrose gradients indicate that the bulk fetal RNA may be different from that of the adult. The base composition of fetal RNA determined chemically is different from the adult, as is the distribution of ^{32}P in the nucleotides of its RNA after alkaline hydrolysis (Table 21.2.1).

The nature of these differences is being investigated along with changes which may occur during development.

21.3 THE MECHANISM OF THE IN VITRO INACTIVATION OF TYROSINE α-KETOGLUTARATE TRANSAMINASE FROM RAT LIVER

D. D. Holten F. T. Kenney

Introduction. – A rapid increase in the rate of synthesis of rat liver tyrosine α-ketoglutarate transaminase is induced by adrenal steroids, with a maximal level of enzyme being obtained 6 hr after injection of the steroid. From 6 to 12 hr after steroid injection the enzyme undergoes degradiation with a half-life of 2.5 hr. Any understanding of the control of enzyme levels in vivo must include an understanding of the mechanism of enzyme degradation as well as enzyme induction, since the steady-state level of the enzyme is a function of both parameters.[1]

We have recently described an in vitro system obtained from the soluble fraction of rat liver[2] which shows an amino-acid-stimulated inactivation of tyrosine α-ketoglutarate transaminase. It was shown that cysteine was required for the inactivation process and that maximal loss of transaminase activity occurred when cysteine and any two of several other amino acids were present. Pyridoxal phosphate was shown to protect the transaminase from amino-acid-stimulated inactivation.

The studies to be reported here show that tyrosine α-ketoglutarate transaminase, freed from its cofactor (pyridoxal phosphate), is unstable and that the amino acids react with pyridoxal phosphate and prevent it from combining with and protecting the enzyme from inactivation.

Results and Discussion. – The amino-acid-stimulated inactivation of tyrosine α-ketoglutarate transaminase was found to be pH dependent and occurred most rapidly at the highest pH measured

(8.85). Pyridoxal phosphate added to the system prevented the amino-acid-stimulated inactivation only for a limited time, after which the transaminase lost activity. These results suggest that pyridoxal phosphate was required for the stability of the enzyme and that at high pH values the amino acids somehow prevented pyridoxal phosphate from exerting this protective effect. Substantiating this conclusion was the report[3] that cysteine, threonine, and histidine, which cause a rapid inactivation of the transaminase under basic conditions, form colorless condensation products with pyridoxal. Extensively purified transaminase (specific activity 14,000) free of pyridoxal phosphate rapidly lost activity in the absence of amino acids. If pyridoxal phosphate was added back to the enzyme and the enzyme exhaustively dialyzed to remove excess pyridoxal phosphate, the enzyme was stable unless amino acids were present. It was concluded that pyridoxal phosphate was required for a stable enzyme and that certain amino acids which form complexes with the cofactor prevent it from stabilizing the enzyme.

The K_m for pyridoxal phosphate protection against tyrosine α-ketoglutarate transaminase inactivation was 7.7×10^{-7} M as compared to a K_m of 3.1×10^{-7} M for pyridoxal phosphate as a cofactor in the transamination reaction. The similarity of the K_m values in the two processes indicates that pyridoxal phosphate protects against inactivation by combining with the enzyme.

The next question to be answered was to determine if the loss of transaminase activity at pH 9 in Tris buffer is due to inactivation or to enzymatic degradation. To answer this question, purified ^{14}C-labeled transaminase was prepared. The purified transaminase (specific activity 14,000) was incubated at pH 9 in Tris buffer until 85% of its activity had been lost. The inactivated enzyme did not react with antiserum for the transaminase. The fact that tyrosine α-ketoglutarate transaminase which was allowed to become inactive over a period of weeks in the freezer could still react with this antiserum indicates that some inactive forms of the transaminase can still combine with antiserum. If the active transaminase was degraded with trypsin until 100% of the activity was lost, none of the transaminase reacted with antiserum. In a control experiment containing trypsin, trypsin inhibitor, and transaminase, the antiserum did react with the transaminase. In no case were any counts solubilized after inactivation of the transaminase in Tris buffer at pH 9. If the transaminase were being extensively degraded in Tris buffer at pH 9, one would expect TCA-soluble peptides to be formed. Also, any contaminating proteolytic enzyme in the purified preparation of the transaminase degrading the transaminase in Tris buffer would have had to be purified along with the transaminase throughout a 415-fold purification. Since this seems unlikely, we feel that the enzyme is being inactivated and not degraded at pH 9. In view of the known ability of proteolytic enzymes to degrade denatured proteins,[4] the physiological significance of the inactivation process may be that inactivation must occur prior to in vivo degradation of the transaminase.

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21.4 INDUCTION OF LIVER TYROSINE α-KETOGLUTARATE TRANSAMINASE BY PYRIDOXINE IN ADRENALECTOMIZED RATS

D. D. Holten F. T. Kenney

Introduction. – Greengard and Gordon[1] have shown that high doses of pyridoxine injected into adrenalectomized rats caused a threefold increase in liver tyrosine α-ketoglutarate transaminase. The induction was reported to be sensitive to puromycin and insensitive to actinomycin D, and it was concluded that the pyridoxine stimulated the synthesis of the enzyme. The authors also reported an increase in the pyridoxal phosphate levels in the liver 1 hr after the administration of pyridoxine.

Since we have shown that pyridoxal phosphate protects the transaminase from inactivation in vitro, it was thought that the increase in transaminase which Greengard and Gordon had observed after pyridoxine injection was due to increased pyridoxal

phosphate levels, which protected the enzyme against degradation. This theory was tested by measuring the rate of incorporation of ^{14}C amino acid into tyrosine α-ketoglutarate transaminase after injection of hydrocortisone or pyridoxine.

Results and Discussion. – Adrenalectomized rats were injected with hydrocortisone 4 hr prior to sacrifice or with pyridoxine 2 and 4 hr before sacrifice. Protein was pulse labeled by injecting each animal with 20 μc of ^{14}C-leucine for 40 min. The transaminase was precipitated from the 105,000 $\times$ *g* supernatant fraction of the cell with antiserum specific for the enzyme, and counts incorporated into the enzyme were measured. Since the period of the pulse labeling was short compared to the half-life of the enzyme, the rate of incorporation of isotope into the transaminase was a direct measure of the rate of its synthesis.[2] The results indicated that with both hydrocortisone and pyridoxine the rate of transaminase synthesis increased to the same extent as the increase in enzyme level. If pyridoxine had increased the enzyme level by increasing the pyridoxal phosphate levels, which in turn had protected the enzyme from degradation, no increase in the rate of synthesis would have been expected. From these results we conclude that pyridoxine increases transaminase levels by inducing an increased rate of synthesis rather than by protecting the enzyme from degradation.

A second experiment was then employed to see if pyridoxine could protect tyrosine α-ketoglutarate transaminase from degradation. A high level of liver transaminase was obtained by injecting adrenalectomized rats with hydrocortisone 6 hr prior to sacrifice. At this time cycloheximide was injected into all rats to inhibit protein synthesis and to exclude any possibility of an induction by pyridoxine. One-half of the animals were then injected with pyridoxine, and the transaminase levels were followed for the next 6 hr. If pyridoxine was able to protect the transaminase against degradation in vivo, those animals receiving pyridoxine would have maintained the high transaminase levels resulting from the hormonal induction while those animals not receiving pyridoxine would have lost the transaminase with a normal half-life of 2.5 hr. The half-life of the transaminase from the animals receiving pyridoxine in this experiment was a normal 2.8 hr, indicating again that pyridoxine does not protect against the in vivo degradation of tyrosine α-ketoglutarate transaminase. Thus, all the data were consistent with the interpretation that an increase in transaminase levels results from an increased rate of synthesis of the enzyme following the injection of pyridoxine.

In contrast to the results of Greengard and Gordon, we have found that the induction of tyrosine α-ketoglutarate transaminase by pyridoxine is sensitive to actinomycin D and therefore requires DNA-directed RNA synthesis. It is hoped that this information will aid in the development of new experimental approaches aimed toward the elucidation of the mechanism of the phenomenon of enzyme induction.

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21.5 ENZYME REPRESSION IN RAT LIVER

W. L. Albritton F. T. Kenney

Introduction. – "Stress," that is, intraperitoneal injection of L-tyrosine or Celite, of the intact animal results in a 10- to 20-fold increase in hepatic levels of the enzymes tyrosine transaminase and tryptophan pyrrolase. This response is completely abolished by adrenalectomy and is replaced in the adrenalectomized animal by a selective repression of the synthesis of tyrosine transaminase. The mechanism of this selective repression is being investigated.

Results and Discussion. – The administration of L-tyrosine or Celite (60 mg per 100 g of body weight) to adrenalectomized rats results in a fall in activity of tyrosine transaminase after a lag of 30 to 40 min. No effect is seen on hepatic levels of tryptophan pyrrolase. This decline in activity is consistent with the known half-life of the enzyme (i.e., 2.5 hr), and it is abolished by previous treatment with actinomycin D (100 μg per 100 g of body weight), indicating the involvement of DNA-dependent RNA synthesis.

That this decline in enzyme activity is due to repression of synthesis and not merely inactivation is being examined employing immunochemical

measurements similar to those described previously[1] and modified by Schimke *et al.*[2] Preliminary results indicate that incorporation of ^{14}C-L-leucine into tyrosine transaminase is abolished, at least at 4 hr after "stress," when the enzyme activity is less than 50% of control and incorporation of label into total soluble protein is unaffected. Measurements are now being made at an earlier time after "stress" when enzyme activity has not yet begun to fall.

Synthesis of tyrosine transaminase can continue unabated for at least several hours when RNA synthesis is abolished by actinomycin, indicating that the template for synthesis of this enzyme is quite stable. Thus the present results provide clear evidence for a mechanism controlling enzyme synthesis at the translational level. The relationship of this translational control to the hormonal induction of enzyme synthesis is being investigated.

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21.6 FRACTIONATION OF RAPIDLY LABELED RNA ON HYDROXYLAPATITE COLUMNS

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Joseph Kendrick

Introduction. – The rapidly labeled RNA of animal tissues consists largely of two types, which differ from one another in base composition and in molecular weight. These are, respectively, a precursor form of ribosomal RNA (pRNA) and an RNA that resembles DNA in base composition (dRNA). For many investigations of regulatory phenomena it would be a distinct advantage if these two types of RNA could (1) be separated from one another and from unlabeled RNA's and (2) be fractionated to reveal individual RNA species within each type. We are investigating the possibilities of using columns of hydroxylapatite for these purposes.

Results and Discussion. – Hydroxylapatite columns were found to have large capacities for binding RNA. Upon elution with phosphate buffers, transfer RNA was readily separated from the high-molecular-weight ribosomal RNA; it thus appears that fractionation on the basis of charge density and/or molecular weight can be easily attained. Phosphorus-32-labeled RNA was prepared from the livers of rats given ^{32}P 20 min before death. Preliminary chromatographic fractionation of this RNA reveals that the bulk of the rapidly labeled RNA is eluted in two discrete peaks. The first of these is eluted with or shortly after transfer RNA, while the second is eluted after most of the ribosomal RNA has left the column. The peaks of radioactive RNA have been collected, and we are currently analyzing the RNA to determine if they differ in base composition. These preliminary observations suggest that hydroxylapatite chromatography may prove to be a most useful tool in fractionating and characterizing ribonucleic acids.

Microbiology

Electron Transport Mechanisms in Microorganisms (22.1, 22.2)

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22.1 CELL MEMBRANE FRACTION OF *STREPTOCOCCUS FAECALIS*

M. I. Dolin

The new naphthoquinone isolated from *Streptococcus faecalis*, 2-solanesyl-1,4-naphthoquinone, occurs entirely in the cell membrane fraction.[1] Determination of the metabolic role of the naphthoquinone therefore requires a better understanding of the composition and function of the cell membrane. The preliminary work[1] concerning the enzyme and cofactor composition of the membrane and cytoplasmic fractions of *S. faecalis* has been extended. Special attention was paid to the compartmentalization of the bound flavin and to the characterization of the flavin by fluorimetric techniques. At least three species of membrane bound flavin can be distinguished, based on the conditions necessary for extraction of the flavin from the membranes with trichloroacetic acid (TCA). Two species of TCA-extractable flavin account for 60% of the total. Liberation of the remaining bound flavin requires treatment of the membrane with proteolytic enzymes prior to extraction with TCA. The flavin released by this treatment has the excitation spectrum and pH-fluorescence behavior of free riboflavin. There is no evidence for the presence of flavin peptides such as those released during enzymic digestion of succinic dehydrogenase.[2]

The membrane contains little or no TCA-extractable DPN; however, treatment of the intact membranes with strong alkali causes the formation of a chromophore which is similar but not identical in fluorimetric behavior to the strong alkali product of DPN.

The presence in the membrane of flavin which can only be released by proteolytic digestion suggests the presence of a covalently bound flavoprotein such as succinic dehydrogenase.[2] The intact membranes, however, have no detectable succinic, malic, or α-glycerophosphoric acid dehydrogenase activity. Of the enzymes examined to date, only one, a DPNH–cytochrome C reductase, is found exclusively in the membrane fraction. Since *S. faecalis* as normally grown is free of cytochrome components, the reductase activity must be regarded as a nonspecific or model reaction. There is as yet only presumptive evidence for naphthoquinone function in cytochrome C reduction by DPNH. The reaction, as catalyzed by membranes or by particle preparations derived from intact membranes, is inhibited by dicumarol and ultraviolet irradiation (360 mμ). Extraction of intact membranes with acetone causes an 84% inhibition of cytochrome C reductase activity.

Available evidence shows that the *S. faecalis* membrane resembles in composition a respiratory particle which is devoid of cytochrome components. From the point of view of comparative biochemistry, such a primitive system could be the nucleus for the formation of a more complete respiratory assembly. The reaction demonstrated with exogenous cytochrome C as oxidant may be a model for the formation of one segment of a complete respiratory chain. In this regard, it is of great interest that certain streptococci, when grown in the presence of heated blood, are reported to contain spectroscopically detectable cytochrome components.[3]

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22.2 DPNH PEROXIDASE

M. I. Dolin

By means of fluorimetric methods it was possible to follow the oxidation of low concentrations of DPNH in the presence of low concentrations of H_2O_2. Under such conditions, steady-state kinetic analysis may be used to discriminate between mechanisms which involve free reduced flavoprotein and mechanisms which involve only binary and ternary complexes of enzyme and substrates.[1] The experiments revealed that the previously determined K_s for H_2O_2 (ref. 2) was artificially high. A portion of the H_2O_2 effect on the velocity of DPNH oxidation is attributable to the fact that H_2O_2 protects the enzyme against inhibition by DPNH. At high concentrations of H_2O_2 (10^{-3} *M*) the enzyme is completely protected.[3]

The experiments permitted an upper limit to be set on the K_s for H_2O_2, but the inhibitions encountered at low H_2O_2 concentrations complicated the kinetics and made it impossible to use the results to predict a mechanism. It was shown, however, that previous data[4] dealing with the effect of substrate structure on the turnover, and on the dissociation and Michaelis constants for reduced pyridine nucleotides, can be interpreted in the following way: DPNH and H_2O_2 combine with the flavoprotein in a random binding sequence to form a ternary complex that dissociates into products in one step.

Recent work[5] has shown that glucose oxidase and xanthine oxidase contain previously undetected fluorescent components. Examination of a DPNH peroxidase preparation revealed the presence of a similar fluorescent substance (emission peak at 445 mμ on excitation at 370 mμ). It appears, therefore, that unrecognized cofactors may be present in a variety of flavoproteins. It is obviously important to characterize these components and to determine their possible function in flavoprotein catalysis.

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Plant Physiology and Photosynthesis

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Plant Physiology (23.1–23.3)

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Photosynthesis (23.4)

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23.1 LEAF SENESCENCE AFTER CESSATION OF GROWTH WITHOUT CELL DIVISION

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D. E. Foard

Introduction. – Studies of plant senescence are complicated by the fact that the main axial organs (said to exhibit "indeterminate growth")[1] themselves produce new organs. In other words, senescence cannot be considered separate from development itself. Certain organs such as leaves (said to exhibit "determinate growth")[1] are structures of limited growth that do not produce new organs. This study explores the usefulness for investigations of senescence of the gamma-plantlet (i.e., a wheat seedling growing without cell division after heavy gamma irradiation of the unsown grain). In this system all organs are determinate; thus the plant embryo becomes analogous to the embryo of a higher animal in which all the organs present in the mature individual at cessation of growth are already present as primordia in the embryo.[2,3] This report describes aspects of senescence in gamma-plantlets after the growth without cell division has ceased. The criterion of senescence used is the deterioration of chloroplasts, whose loss of integrity is indicated by decreasing chlorophyll content per unit tissue despite favorable illumination. This is a characteristic of senescence of green tissue generally[4] and of wheat leaves in particular.[5,6]

Results and Discussion. – Intact gamma-plantlets (700 kr) were compared with unirradiated controls with respect to the time course of senescence of apical portions of the first foliage leaf. Since this organ is initially present at the time of irradiation and is normally determinate, we may consider that even in unirradiated plants it is "programmed to senesce."

The gamma-plantlet leaf reaches its final length (19 mm) about 13 days after sowing; the apical portion then slowly loses chlorophyll with an apparent half-life of about 6 or 7 days. Unirradiated

control leaves reach a final length (about 215 mm) about 16 days after sowing; the apical portion of the control leaves then slowly loses chlorophyll, with a half-life of about 8 or 9 days. Thus there is no precipitous deterioration of the gamma-plantlet leaf but rather a slow senescence roughly comparable with the senescence of unirradiated leaves after growth has ceased. When apical portions that had already stopped growing were cut from 10-day-old gamma-plantlet and unirradiated leaves and floated on water, the time courses of their chlorophyll loss were the same, with a half-life of about 6 days. Addition of benzimidazole greatly retarded senescence of the detached leaf tips; kinetin gave almost as much protection, and Ni^{2+} salts gave very slight protection against senescence. The effectiveness of each of these three senescence-retarding chemicals was similar in gamma-plantlet and unirradiated leaf tips floated in vitro. The similar courses of senescence in gamma-plantlet and unirradiated control leaves indicate that the gamma-plantlet can be useful biological material for studies of plant senescence uncomplicated by other aspects of development.

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23.2 STUDIES OF GERMINATION AND OF X-RAY-INDUCED ESR SIGNALS IN LETTUCE SEEDS AFTER HYDRATION AND REDRYING

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Introduction. – With lettuce seeds, radiation-induced dormancy[2] can be studied after doses that induce readily detectable ESR signals.[3] Moreover, lettuce seeds can be hydrated and redried without loss of viability.[3] This last property of lettuce seed permits the introduction of experimental parameters, such as chemical treatments, not otherwise possible with combined biological and radiological studies of dry material. The ultimate usefulness of this technique depends on the extent to which the hydration followed by redrying alters subsequent biological and radiological properties of the seeds. In this study we have soaked and subsequently redried lettuce seeds and afterwards looked for changes in germination and growth as well as for changes in concentration and characteristics of native and x-ray-induced ESR signals.

Results and Discussion. – When lettuce seeds are placed on moistened filter paper at 25°C, their water imbibition is completed within a few hours. For the New York lettuce seeds studied, germination under 25 ft-c of white light occurred between 14 and 20 hr after sowing, the time required for half the seeds to germinate being about 16 to 17 hr. Wet seeds can be redried within 8 to 24 hr by putting them on filter paper in a Büchner funnel attached to a flask under vacuum for 1 min followed by transferring to a desiccator containing Drierite. Seeds were so dried 0, 1, 2, 3, 6, 9, or 12 hr after sowing. After such redried seeds were reimbibed, we studied the following biological characteristics: final germination percentage, time course of increase in germination percentage, and average root length at a given time (35 hr) when essentially all the seeds had germinated. For the seeds initially imbibed for 12 hr, the time for half the seeds to germinate was lengthened to 21 hr; neither the final extent of germination nor root growth was significantly reduced by the 12 hr imbibition followed by redrying. For the shorter times between imbibition and redrying we observed no changes in any of the three biological measurements.

In parallel studies also with New York lettuce, the seeds were completely wetted by infiltration and steeping for $1\frac{3}{4}$ hr, redried, and exposed to 0, 200, or 800 kr of 150-kvcp x rays. The single central ESR signal in all these seeds had the same width (6.5 oersteds peak to peak of the derivative curve), *g* value (2.006), and overall relaxation time as indicated by power saturation measurements. After 200 and 800 kr, the derivative signals were about 2.3 and 8.4 times as large as for the unirradiated seeds. For any given exposure, including the

unirradiated controls, the shape, *g* value, and relaxation time of the ESR signals were not appreciably altered by the hydration followed by redrying, but the signal size may have been slightly less in the seeds so treated. Even imbibed seeds that had been killed by boiling for 15 min showed no differences in background or x-ray-induced ESR signals after they were redried. These results indicate that the complete wetting of lettuce seeds followed by redrying does not greatly influence biological or radiological properties of these seeds. This technique will therefore permit the performing of experiments not otherwise feasible with biological material in the dry state.

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23.3 HISTOLOGICAL STUDIES OF MITOSIS IN DORMANT LETTUCE SEEDS

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A. H. Haber

Introduction. – Lettuce seeds whose germination (i.e., macroscopically detectable expansion of the embryo beyond mere water imbibition) is prevented by temperatures slightly above the upper limit that permits germination are said to be "thermodormant." Despite the absence of visible growth of the embryo, in certain batches of lettuce seed mitosis and cytokinesis can occur in the thermodormant seeds.[1] Observations on extent but not precise locations of mitotic activity in such seeds have come from squashes of the apical approximately 0.5 mm of the radicle (hypocotyl-root axis) of embryos dissected from seeds. The question whether microscopically detectable elongation occurs, like the question of precise locations of the mitoses, cannot be answered from squashes alone. An experiment was performed, therefore, employing concurrently the technique of squashing and that of sectioning so that results obtained by each method could be directly compared. This permits us to see whether any microscopically detectable elongation of the embryo axis occurs and to see the precise locations of the mitoses in the thermodormant embryos.

Results and Discussion. – Lettuce seeds from a single batch of the New York variety were sown on moistened filter paper in petri dishes at 29°C. Seeds were fixed on each of the first four days following sowing; on each of these days one group of seeds in the sample was prepared for squashing, and another group of the same sample was prepared for sectioning. Since the purely physical process of water imbibition produces some swelling, the embryo axis length of the thermodormant lettuce seed is more appropriately compared with the embryo axis of a seed that is hydrated than compared with the embryo axis of a dry seed. For controls, therefore, some dry seeds were cut transversely midway through the cotyledons and were vacuum infiltrated with 0.1 *M* sodium azide for 1 hr. The seeds were then transferred to fresh azide solutions and allowed to imbibe for 24 hr. These seeds were then prepared for sectioning.

Comparison of thermodormant seeds and azide-imbibed seeds shows no difference in axis length. Thus in the thermodormant seeds there is no overall elongation of the embryo axis as a whole. Moreover, examination of serial sections of 20 of 36 two-day-old thermodormant seeds shows no evidence of localized elongation in the embryo axis in the absence of germination. Mitoses were present in 3 of these 20 seeds. Thus, these three plants demonstrate that mitoses can occur in thermodormant seeds in the absence of overall elongation of the axis and in the absence of localized elongation anywhere within the axis.

In the remaining 16 of 36 plants observed there was evidence that some localized elongation in the embryo axis had occurred, even though there was no elongation of the axis as a whole and thus no germination. This elongation could be detected in three ways: (1) crushing of the cap of the radicle against the surrounding seed coats, (2) crushing of single cells or a small number of cells, and the abnormally large size of cells adjacent to the crushed ones, and (3) bulges in the vascular cylinder. Of the 16 thermodormant seeds in which localized elongation could be detected, 13 had mitoses in them. Four of these plants had mitoses only in abnormally large cells, three had mitoses only in normal-size cells, and six had mitoses in both types of cells. Thus the occurrence of mitoses is not correlated with either localized elongation within the axis or overall elongation of the axis as a whole.

In this experiment, mitoses were not observed in squashes or sections of the thermodormant seeds until the second day after sowing; by the third day after sowing, mitoses were again absent or negligible. Examination of squashes of the apical approximately 0.5 mm of the radicle of thermodormant seeds two days after sowing revealed that mitotic figures were present in 10 of 20 plants observed. The distribution of figures among these 10 radicles was as follows: 1, 3, 3, 4, 4, 27, 30, 33, 35, 51. Examination of sections of the entire embryo of thermodormant seeds after sowing revealed that mitoses were present in 16 of 36 plants observed. The distribution of figures among these 16 embryos was as follows: 1, 2, 2, 2, 2, 6, 7, 9, 9, 9, 10, 22, 27, 34, 39, 75. Of these 256 mitoses, 228 (or about 89%) occurred within the apical 0.5 mm of the radicle. The other 28 mitoses were located more basally in the hypocotyl. No mitoses were found in the shoot apex, leaf primordia, or cotyledons. The results obtained from examination of both squashes and sections, then, are in good agreement with regard to number of mitoses, their time of appearance, and their histological location in these thermodormant lettuce seeds. In the absence of both localized elongation within the axis and overall elongation of the axis, the mitoses in the radicles of thermodormant seeds occurred in all the primary tissue meristems in a pattern similar to that in the apex of growing roots. Elongation would appear, therefore, unnecessary for the pattern of distribution of mitoses in the primary meristems of the growing root.

These studies of thermodormant seeds have demonstrated the following: (1) Mitoses can occur in the absence of any elongation. (2) Although localized elongation can occur in some seeds, there is no elongation of the embryo axis as a whole in any of the seeds; in such seeds, mitoses may occur in regions of localized expansion as well as in regions that have undergone no elongation. (3) Mitoses occur mainly in the apical 0.5 mm of the radicle and are distributed in the primary tissue meristems in a pattern similar to that in growing roots; thus the normal pattern of distribution of mitoses in the root can occur in the absence of concurrent elongation of the root.

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23.4 PHOTOSYNTHESIS

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For some years our laboratory has been involved in trying to understand the energy conversion steps of photosynthesis. This work has been described in previous semiannual reports, and we shall not give this background information here. In the last six months we have concentrated on two aspects of the millisecond delayed-light emission from the functionally active chlorophyll of whole algal cells: (1) emission at low temperatures (0 to −130°C) where no chemistry may take place, and (2) emission from cells poisoned with 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU), in which a limited amount of chemistry may take place. These experiments imply that at least two mechanisms must be involved in the presumed recombination of electrons and holes which leads to delayed light emission, and that one mechanism is nearly independent of temperature.

Low-Temperature Observations. – Tollin, Fujimori, and Calvin[2] reported the presence of measurable delayed-light emission at −100°C and even lower temperatures after illumination of cells with a single flash of light. This observation seemed to contradict our experience at temperatures above freezing, which indicated a strong temperature dependence of delayed-light emission under all experimental conditions. Our delineation of an activation energy of 0.5 ev for delayed-light emission involving the reaction center of the oxygen-evolving system of photosynthesis[3] would seem to imply that no delayed light should be observed at low temperatures.

We have now repeated and extended the low-temperature observations of the Berkeley group. The emission which they reported is indeed present, but it is ephemeral. It is best observed after a single flash of illuminating light, and it disappears after successive flashes or after 15 sec of continuous illumination. After the emission has been "burned out" by illumination, the ability to emit delayed light returns after about 5 min of darkness. We interpret this "burnout" phenomenon as the charging up of reaction centers within the chlorophyll system due to inability of the electronic charge to leave the chlorophyll system at low temperature. Once the reaction centers are charged, they may no longer separate electrons

and holes, and the low-temperature delayed-light emission has "burned out." This interpretation implies that enzymatic steps or other reactions involving movement of nucleons are involved in the normal flow of charge out of the chlorophyll system. Apparently, charge may slowly drain off the reaction centers at low temperature in the dark so that after 15 min the frozen cells are able to produce another burst of delayed light.

The very presence of the delayed-light emission at –100°C implies that temperature-independent charge migration may take place within the chlorophyll system. Such migration would probably involve solid-state tunneling processes. Such processes presumably occur also at room temperature, but the limiting factor at higher temperatures appears to be the number of electrons which are available to the chlorophyll system, a quantity which is modulated by the enzyme systems of photosynthesis. Thus our 0.5-ev activation energy would only be observed at high temperatures when charge is allowed to leave the chlorophyll system.

The time kinetics of decay at low temperature may be contrasted to the kinetics observed above freezing. At room temperature, anything which affects the enzymatic steps (poisons, mutations, temperature) has a concomitant effect on the kinetics of delayed-light emission. On the other hand, nothing we have so far tried has any strong effect on the kinetics of delayed-light decay at low temperatures. This observation is in agreement with the hypothesis which suggests a different mechanism for delayed-light emission in the two types of experiment.

By contrast, the intensity of delayed-light emission at –100°C is dependent on various experimental parameters. A mutant of *Scenedesmus*, in which the oxygen-evolving system of photosynthesis is nonfunctional, emits no delayed light at either high or low temperatures. Thus all delayed-light emission apparently requires the ability to separate charge at the reaction center which leads to the oxygen-evolving chemistry. The mirror image of this mutant, a strain of *Scenedesmus* in which the pyridine-nucleotide-reducing reaction center is nonfunctional, emitted 30 times more delayed light at –100°C than the wild type. This observation implies that energy migration may take place from one photochemical reaction center to the other. Such an implication is in complete disagreement with the presently accepted scheme of the first steps, which involves separate pigment systems for the two different reaction centers, one leading to oxygen evolution and the other leading to pyridine nucleotide reduction. From our observations we suggest that only a single pigment system is involved, with spatially separated reaction centers for the two types of chemistry.

Poisoning with CMU, which we interpret from experiments at room temperature to result in a block of electron flow out of the oxygen-evolving reaction center, results in a reduction of delayed-light emission at low temperatures. We interpret this as due to the presence of fewer uncharged reaction centers in the presence of CMU at the time the cells are frozen. This seems reasonable because CMU also reduces the 1-msec delayed-light emission at room temperature. However, CMU poisoning results in a much increased 100-msec delayed-light emission at room temperature so that the reaction centers must not become fully charged at room temperature in the presence of CMU. We interpret this as involving a temperature-dependent charge migration in the chlorophyll system, a process which cannot occur at –100°C.

Emission from Cells Poisoned with CMU. – In cells poisoned with CMU the effect of temperatures in the biological range (10 to 40°C) fits the Arrhenius equation. The slope of the curve measures an activation energy of 0.5 ev, which we take to be the energy gap between the first excited singlet of chlorophyll (from which the light is emitted) and the first enzyme which accepts electrons from the chlorophyll system. We are therefore taking some care to define this slope and to determine whether various experimental parameters affect the slope. The parameters which we have so far tested (various poisons, various plant phyla, measurement times from 1 to 80 msec, various intensities and wavelengths of exciting light) have had no effect on the activation energy. This result is in agreement with the hypothesis that this is indeed an activation energy for one of the functional chlorophyll-enzyme interfaces.

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NIH-AEC Cooperative Projects

CARCINOGENESIS PROGRAMS (24.1–24.21)

Biochemistry of Carcinogenesis (24.1–24.3)

Stanfield Rogers
J. H. Evans
R. R. Long
D. W. Parsons
T. J. Stephens, Jr.
Martha S. Taylor
Peggy H. Thomas

Enzymology of the Carcinogenic State (24.4–24.7)

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F. T. Kenney[a]
Marialuisa Melli[a,b]
Lawrence Rosen[a]
Shigemi Iiyama [a,b]
E. L. Murray[a]
L. E. Roberson[a]

Mammalian Chemical Carcinogenesis (24.8)

A. C. Upton[a]
N. K. Clapp[a]
A. W. Craig[a,b,c]
Mildred G. Hayes[a]
R. E. Toya, Sr.[a]

Chromosomal Effects of Chemicals and Radiation (24.9)

E. H. Y. Chu[a]
J. E. Trosko[a,d]
Margaret R. Kasschau[a]

Carcinogen Biology (24.10, 24.11)

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Karen B. Lane[a]

Effects of Radiation and Chemicals on Paramecium (24.12)

R. F. Kimball[a]
E. G. Bailiff[a]
Barbara R. Bussell[a]
Carrie L. Wells[a]

Molecular Biology of Carcinogenesis (24.13, 24.14)

F. J. Bollum[a]
B. K. Zimmerman[a]
Giovanni Cassani[a,b]

Chemical Mutagenesis in Microorganisms (24.15)

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Inhalation Carcinogenesis (24.16–24.21)

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B. E. Sise
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M. A. Turner
J. R. Webb
Mary Lou Williams

ULTRACENTRIFUGE DEVELOPMENT PROGRAM (24.22–24.25)	
Tumor Virus Isolation Studies (NCI) (24.22, 24.23)	**Respiratory Virus and Viral Subunit Isolation (NIAID) (24.24, 24.25)**
N. G. Anderson[a] W. D. Fisher[a] K. W. Allen[f] Mary Lee Barber[f] E. L. Candler, Jr. Tom Grizzard[a] C. T. Rankin, Jr. Elizabeth L. Rutenberg[f]	N. G. Anderson[a] P. A. Van Dreal[f] R. E. Canning[a] G. B. Cline[f] Margaret T. Guess Charlotte A. Langer[i]
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Carcinogenesis Programs

24.1 A QUALITATIVE METABOLIC DIFFERENCE BETWEEN HUMAN SQUAMOUS CELL CARCINOMA OF THE CERVIX AND NORMAL CERVICAL SQUAMOUS EPITHELIUM

Stanfield Rogers Peggy H. Thomas
A. W. Diddle[1] R. R. Long

Studies of the metabolism of the epithelium of normal cervix revealed a rather uniform uptake of all the amino acids in T.C. Media 199 (Difco), but the uptake varied in quantitative degree, as have other tissues studied.[2] In studies of carcinoma of the cervix derived from two individuals, one of which was repeatedly tested using tissue from the cervix as well as from more peripheral local extensions, a single qualitative difference was found. The cancer cells excreted large amounts of the amino acid lysine into the media. There were several major quantitative differences involving other amino acids but no other qualitative. This single qualitative difference becomes more striking when it is realized that mammalian cells have no known potential to synthesize this wholly essential amino acid.[3] Since this ability might well be due to information carried into the cells by an infecting virus, efforts are being made to accumulate cervical carcinoma tissue for virus and DNA extraction for test in culture systems using the ability to synthesize lysine as a biochemical marker for the presence of the hypothetical virus. Such a test system may provide the only way that one can test the significance of viruses and virus-like particles found in human tumors without infecting man himself.

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[1]University of Tennessee Memorial Hospital, Knoxville.

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24.2 THE SHOPE PAPILLOMA VIRUS: A PASSENGER IN MAN AND ITS SIGNIFICANCE TO THE CONTROL OF THE HOST GENOME

Stanfield Rogers Peggy H. Thomas
R. R. Long

Previously reported studies have apparently indicated that the information for the synthesis of the arginase induced by the Shope virus in rabbit epithelium is derived from the viral genome rather than being rabbit information.[1] In some unrelated studies not yet published in detail,[2] it has been found that rabbits infected with the virus have a

relatively low blood arginine. It has been recently reported[3] that this unique arginase[1] can be used as a marker in tissue culture systems even though evidence for protein coat synthesis cannot be demonstrated. On this basis it seemed worthy of test to find whether people who had worked with the virus in the laboratory might have become infected with the virus and have a low blood arginine and neutralizing antibodies against the virus.

The first tests were with the sera of 17 individuals who had worked with the virus over the past 35 years as compared with the sera from 21 normal controls, 19 of which were patients with various diseases randomly selected from patients in the University of Tennessee Hospital at Knoxville. Seven of the individuals who had worked with the virus had arginine levels very different from the controls. Several others were in the lowest known range of the norm of both our normal controls and that in a very sizable literature. The tests were next run "blind" with 14 sera provided through the cooperation of Dr. C. A. Evans, University of Washington School of Medicine, Seattle. Seven of these were from individuals who had worked with the virus and seven from normal controls selected by them and intermixed. Of this group five sera were either borderline or well below the levels found in the controls. All the controls were well within the known normal range.

Standard neutralization tests of 14 of the sera of individuals with a history of working with the virus yielded two positive inhibitory sera on virus infectivity. It would appear possible that human cells infected with the virus made no protein coat antigen, much like the tissue-culture-infected cells, and much like the cells of the domestic rabbit, which make only small amounts of protein coat antigen. These two sera were from individuals who had been working for some time with virus purification, who would have had the opportunity to get repeated sizable inoculations with the virus to supply the protein coat antigen. On the other hand, immunodiffusion tests of 12 sera of individuals working with the virus against the purified enzyme derived from domestic rabbit papillomas yielded eight which gave positive bands. There was no cross reaction against purified rabbit liver arginase.

It thus appears that a considerable proportion of people who have worked with this virus have become infected, resulting in no clinical symptomology but resulting only in antibodies against the virus-derived enzyme and significantly low blood arginines. In view of the excellent health of these individuals over a long period of time, it seems that the papilloma virus might be used to infect man to provide a low blood arginine in individuals having genetic imbalances which would be thereby corrected. It is hoped that there are other passenger viruses in man which can be found to carry the information for the synthesis of enzymes and which could be used in an analogous way to genetically cure or palliate various diseases including those caused by genetic deletion.

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24.3 EFFORTS TO TRANSMIT SYNTHETIC GENETIC INFORMATION USING THE SHOPE VIRUS DNA AS A CARRIER

Stanfield Rogers Peggy H. Thomas
R. R. Long

The rationale of this study was described in the previous progress report.[1] Together with Fred Bollum of the Molecular Biology Group, efforts were made to attach the polynucleotide, polyinosine, onto the Shope virus DNA using an end-terminal addition enzyme. These efforts have thus far failed. This seems most likely due to the cyclic nature of the virus DNA helix. That the linear helix was not infective was reported this spring by Vinograd. Since it seems possible that a linear helix tightly coiled might prove infective, these studies will be continued despite the lack of an enzyme not yet demonstrated, but certainly existent, which specifically breaks the cyclic helix of the virus and another enzyme which puts it back together – perhaps not unlike the end-terminal addition enzyme. When such enzymes

are found, they will make such a problem the more feasible.

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24.4 HORMONAL ENZYME INDUCTION IN THE REUBER HEPATOMA IN VITRO

F. T. Kenney
R. L. Tyndall[1]
Joseph Kendrick
L. E. Roberson

Introduction. – The Reuber hepatoma is a minimal deviation tumor of rat liver that was placed in culture several years ago by Morse and Potter. The cultured tumor retains the ability to respond by stimulation of enzyme synthesis to adrenal steroid hormones,[2] and thus represents an essential stage of intermediate complexity in which hormone action can be analyzed. In addition, mechanisms of growth regulations in normal liver can be compared to those operative (or inoperative) in this neoplastic "mutant," thus giving insight into the nature of neoplastic growth. We have initiated a study of transaminase induction in these cultures, in order to define the various parameters involved and to compare the response to that occurring in the liver in vivo.

Results and Discussion. – Addition of hydrocortisone (10^{-6} *M*) results in an increased level of tyrosine transaminase in the hepatoma cells. The increase begins after 3 to 4 hr and continues until a plateau is reached after 12 hr. The response is thus about one-half as fast as observed in vivo. Kinetic considerations suggest that this reflects a slower enzyme turnover in the tumor. This was confirmed by adding puromycin after a high enzyme level was reached; with enzyme synthesis blocked, the transaminase level fell with a half-life of about 6 hr. Half-life of the transaminase in vivo is about 3 hr.[3] It is of interest that the hepatoma cells were able to metabolize puromycin vigorously; thus, repeated additions of high levels of the antibiotic were necessary to block protein synthesis. In this respect the cells are similar to liver and not like other cultured tumors (HeLa, L cells, etc.) wherein low doses of puromycin are effective, a result underscoring the "minimal deviation" nature of the Reuber hepatoma.

Actinomycin (10^{-6} *M*) blocked enzyme induction by hydrocortisone. As in the liver, there is an increase in RNA synthesis after adding the hormone. Preliminary experiments indicate that this response is limited in magnitude. Further work of this nature is planned, as it may aid in resolving the discrepancy between the general response in RNA synthesis and the specificity of enzyme induction, that we observe in vivo.[4]

As discussed previously[5] the major difference in the response to hormone that has been detected[2] is the failure of the induced enzyme level to fall after the peak has been reached. This fall has been attributed to restoration of the synthesis of a cytoplasmic repressor after the administered steroid is metabolized.[6] The tumor cells thus appear to be unable to limit the response to steroid, perhaps because they are unable to metabolize the hormone and thus permit repressor formation. The capability of the hepatoma cells for enzymatic degradation of hydrocortisone is now being studied.

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24.5 FURTHER STUDIES ON PROTEIN SYNTHESIS BY PREPARATIONS FROM LIVERS OF THE RAINBOW TROUT

Lawrence Rosen
C. C. Campbell III[1]
G. D. Novelli

Introduction. – The earlier work[2] related to protein synthesis in normal rainbow trout has been extended.

Results and Discussion. – The incorporation of phenylalanine into polypeptide by two classes of

ribosomes in the presence of a high-speed supernatant and cofactors has been further studied. These classes of ribosomes are obtained from a post-mitochondrial supernatant (PMS). The PMS is treated with deoxycholate and then layered on a discontinuous sucrose density gradient composed of 0.5 *M* sucrose on either 1.0 *M* sucrose or on 2.0 *M* sucrose and subjected to a high-speed centrifugation. A ribosomes are obtained from the former and C ribosomes from the latter density gradient.

Under optimal conditions, the endogenous incorporation of phenylalanine is essentially complete in 60 min for both classes of ribosomes. The level of endogenous incorporation for C ribosomes is approximately 15 times that for A ribosomes.

With respect to the increase of phenylalanine incorporation stimulated by the addition of poly U at zero time, it has been found that the A ribosomes are stimulated to a much greater relative extent (15-fold) than are the C ribosomes (1- to 3-fold). A preliminary analysis of the kinetics of the poly U stimulation indicates that for the A ribosomes, the increase is mainly due to the interaction of poly U with monomers which are devoid of endogenous template (increase I).[3] For the C ribosomes the stimulation appears mainly due to interaction of poly U with ribosomes which have been released from endogenous template following chain completion (increase II).[3] These results are consistent with the working hypothesis that a population of ribosomes is composed of (1) members which can be activated by the addition of template RNA and (2) another group which are bound to endogenous template. Following chain completion by this latter group, monomers are released which are capable of interacting with added template RNA.

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24.6 IN VIVO STUDIES OF PROTEIN AND RNA SYNTHESIS IN TROUT LIVER

E. L. Murray G. D. Novelli

Introduction. – After studying the various parameters of the in vitro amino-acid-incorporating system from the livers of rainbow trout,[1] we turned our attention to in vivo studies in order to develop some normal parameters regarding the synthesis of protein and nucleic acid to be applied to anticipated studies with hepatomatous fish.

Results and Discussion. – The first problem encountered was that of selecting the route of injection of the labeled precursor. In studies of the in vivo rate of protein synthesis, we injected ^{14}C-phenylalanine intraperitoneally since this route had been quite successful with both rats and mice. With the fish, however, results from one fish to the next were quite erratic, and we suspected variations in uptake from the body cavity. In order to obtain comparable pool sizes in different animals we thought that a direct injection into the heart should give similar liver pools with different fish. Here again uptake seemed to be poor and results erratic. We then learned that the circulatory system in the trout is from the heart through the gills and back to the liver. As the radioactive amino acid passed through the gills, much of it was excreted, and in addition we learned that gill tissue contains a potent amino acid deaminase. We finally succeeded in obtaining nicely reproducible results by injecting the isotope into the dorsal artery between the third and fourth gill arches.

For in vivo protein synthesis five fish for each time point were injected with ^{14}C-phenylalanine (1.6 μc per 100 g of body weight), and fish were sacrificed 30 min, 45 min, 1 hr, 2 hr, 3 hr, and 4 hr postinjection. The livers were excised, weighed, and homogenized in 2.5 volumes of medium A using a Teflon-glass homogenizer. The homogenate was centrifuged 30,000 $\times$ *g* for 15 min (S-30), and specific radioactivity of the protein was measured in the usual manner. The data are expressed as counts per minute per milligram of protein. The ^{14}C-phenylalanine is rapidly incorporated into liver soluble protein for the first hour, then levels off, and dips slightly during the second hour. These results are quite similar to those seen in the intact rat. In a comparative experiment involving a number of assumptions, rate calculations indicate that the rainbow trout living at a

temperature of about 11°C is making liver protein at about the same rate as the rat is at 37°.

For the RNA studies, similar injections were made, except that the isotope was ^{14}C-orotic acid. We studied the RNA fractions – nuclear and cytoplasmic. Nuclei were isolated according to Kosow[2] and suspended in medium A. The S-30 fraction was used to measure cytoplasmic RNA. The RNA was precipitated with 10% TCA–1.0 *M* NaCl. The pellets were carried through a lipid extraction, and the RNA was hydrolyzed with alkali. The amount of RNA and its specific radioactivity were determined on the acid-soluble fraction after alkaline degradation.

The specific radioactivity of nuclear RNA is very high soon after injecting the isotope and continues to increase for about 3 hr, after which the specific activity declines.

The specific activity of the cytoplasmic RNA is much lower than that of nuclear RNA, but continues to rise steadily for at least 5 hr. It is clear that RNA is synthesized in the nucleus and then goes into the cytoplasm. We were able to calculate that most of the radioactive RNA lost from the nucleus during the third and fourth hours could be found in the cytoplasmic RNA.

We expect soon to carry out similar studies with hepatomatous fish.

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24.7 STUDIES ON RIBOSOME SYNTHESIS IN RAT LIVER

Marialuisa Melli Shigemi Iiyama
G. D. Novelli

Introduction. – A previous study of the effect of puromycin on RNA synthesis in the livers of intact rats revealed the following:[1]

1. Nuclear RNA *synthesis* (as measured with a 10-min pulse of ^{14}C-orotic acid) is uninfluenced during at least 7 hr in the absence of protein synthesis (brought about by hourly injection of puromycin – 10 mg per 100 g of body weight).
2. Nuclear RNA *accumulation* (measured by a 2-hr labeling with ^{14}C-orotic acid) is also normal in the puromycin-treated animals.
3. Transfer-like RNA (4S) appears also to be normally synthesized in the treated animals.
4. Ribosomal RNA (prepared by phenol extraction and measured as 28S and 18S) showed a normal rate of labeling with ^{14}C-orotic acid for the first 2 hr of puromycin treatment, but appeared to cease completely thereafter. Since the rate of synthesis of RNA was unimpaired in the treated animals and was apparently not transferred to the cytoplasm nor accumulated in the nucleus, it seemed that in the absence of protein synthesis the RNA broke down as fast as it was synthesized.

In recent experiments, by improving the rapidity of the phenol extraction, we have been able to show that during the period of inhibition of protein synthesis, labeled 28S and 18S RNA's are present in the postmitochondrial supernatant (PMS) of the liver homogenate. This suggests that in rat liver in the absence of protein synthesis, the situation is comparable to the one described in chloramphenicol-treated *E. coli*, where RNA synthesis is independent of protein synthesis.

Results and Discussion. – Protein synthesis was kept completely inhibited in rats by hourly injection of puromycin (10 mg per 100 g of body weight). The rats were injected with ^{14}C-orotic acid 2 hr before sacrifice, and RNA was extracted from the PMS, the microsomes, the ribosomes, and the 100,000 × g supernatant fraction of the liver homogenate using the phenol-dodecylsulfate procedure. The $O.D._{260}$ profiles of the RNA extracted from all the above fractions appeared to be the same in treated and control animals except when the RNA was extracted from the 100,000 × *g* supernatant. In this fraction control animals showed only a single peak of $O.D._{260}$ in the 4S region, whereas the treated animals had in addition to the 4S peak, two small peaks in the 28S and 18S region. This is due to the fact that in the treated animals the ribosomal population is composed mainly of ribonucleoprotein particles lighter than monoribosomes that do not sediment completely during 4 hr of centrifugation at 100,000 × *g*.

The radioactivity profiles of the extracted RNA appears to be normal in the treated animals, showing 28S, 18S, and 4S peaks. However, in the treated animals the specific activity of these peaks was lower than in the controls. The extent of inhibition seems to be different in different experiments, ranging from normal values to 50–60% inhibition. This variability is probably due to the extreme lability of the RNA fraction in the treated animals. In fact, labeling of these fractions in treated animals could be shown only if the RNA was extracted immediately after sacrifice of the animals. If the fractions of the liver homogenate were kept frozen and then extracted, the specific activity appeared to be extremely low and the labeling appeared in the light region of the gradient, between the 18S and 4S peaks. In order to determine whether this labile fraction of cytoplasmic RNA in the treated animals had the same specific activity as that of the stable fractions from control animals, the PMS will be digested with alkali and the resulting 2′–3′ nucleotides will be separated by column chromatography from the 5′ nucleotides of the acid-soluble pool.

We have previously observed[1] that animals under puromycin-induced inhibition of protein synthesis could recover the ability to synthesize protein within 4 to 6 hr if no further injections of puromycin were made. Under such conditions preliminary experiments using double labeling (^{14}C-orotic acid during puromycin treatment and ^{3}H-orotic acid during the recovery period) indicate that the labile RNA synthesized during puromycin is not utilized for ribosome synthesis for up to 7 hr after the last injection of puromycin.

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24.8 PATHOLOGIC CHANGES IN MICE TREATED WITH DIALKYLNITROSAMINES[1]

N. K. Clapp A. W. Craig

Dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) have both been shown to be potent liver carcinogens in the rat.[2,3] However, in the mouse, preliminary reports have suggested differences in the organ specificity of these drugs; DEN administration has produced liver tumors,[4] whereas DMN has produced lung tumors.[5] In this investigation, mice treated with nitrosamines administered daily in the drinking water were examined for precancerous changes.

Results and Discussion. – Male RF mice (56–70 days old) were treated for a period of 7 weeks. DEN was given at a concentration of 8 mg per 100 ml of drinking water, and DMN was administered at 1 mg per 100 ml of drinking water. Animals were sacrificed at weekly intervals for the first 10 weeks and at 12, 14, and 16 weeks after treatment was begun. Tissues were preserved in Zenker-Formol fixative and examined microscopically.

DMN-treated animals occasionally became ascitic, but no animals died. Early degenerative changes were first observed in the centrilobular areas of the liver after 3 weeks of treatment, and centrilobular necrosis was apparent by the fourth week. A perivascular lymphocytic infiltration and a slight granularity in the gross appearance were observed simultaneously. These histologic changes were observed throughout the period of drug administration. Necrosis of the liver was markedly reduced one week after stopping DMN administration, but perivascular cuffing remained evident through the 12th week of the experiment. Regeneration of the hepatic parenchyma was virtually complete at that time. Although no differences were found between control and treated animals in the first 14 weeks, multiple pulmonary adenomas were found in those animals sacrificed after 16 weeks.

The DEN animals became progressively dehydrated and emaciated during the 7 weeks of drug administration, with 40% lethality. The onset of centrilobular degeneration and centrilobular necrosis paralleled chronologically that observed following DMN treatment, but pleomorphism of parenchymal cells was also seen. The surfaces of the livers were granular, beginning at 4 weeks, with the necrosis progressively increasing in severity. Hyperplasia of small oval cells was first observed 5 weeks after continuous DEN administration. These cells appeared similar morphologically to bile duct epithelial cells. The small oval cells increased in number, aligned themselves in rows, and coalesced to form ductlike structures of bizarre shape at 8 weeks. The hyperplastic changes regressed 2 weeks after cessation of DEN administration, and a disorganized and incomplete re-

generation followed; some ductlike structures persisted through the 16-week experimental period. Eosinophilic inclusions were first observed at 6 weeks in the nuclèi of hypertrophied hepatic cells, but hypertrophied parenchymal cells and eosinophilic inclusions were seldom seen at 12 weeks. No tumors were found in the DEN-treated animals; however, information from related studies shows that hepatomas are induced in RF mice after a longer induction period.

The minimal morphological change that preceded the rapid development of pulmonary adenomas in the DMN-treated mice contrasted with the more complicated sequence of changes in the liver. Although the degenerative changes in the liver caused by the two nitrosamines were similar initially, the subsequent development of "oval cell" hyperplasia was found only in DEN-treated animals. This, together with the presence of eosinophilic inclusions in the nuclei of hypertrophied hepatic cells, may possibly be indications of preneoplastic changes.

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24.9 EFFECT OF ULTRAVIOLET RADIATION ON DNA SYNTHESIS IN SYNCHRONIZED MAMMALIAN CELLS IN VITRO

E. H. Y. Chu J. E. Trosko

Introduction. – Differences in uv sensitivity, as measured by induced chromosome aberrations[1] and cell survival,[2] have been shown to exist during different cell stages in Chinese hamster cells. Since it has been shown that uv-induced thymine dimers can interfere with normal DNA synthesis in bacteria[3] and in a polymerase system in vitro,[4] and since uv can induce thymine dimers in the hamster cells,[5] it is of interest to study the possible interrelationship between the uv-induced thymine dimers, uv effect on DNA synthesis, and other uv-induced biological changes. This report summarizes the work on uv inhibition of DNA synthesis in these cells.

Methods. – Cells were treated with 5-aminouracil for 24 hr. After rinsing and reincubation in fresh standard medium, most cells resumed DNA synthesis and reached a peak mitotic activity at the 8th hour.[6]

In the first series of experiments, cells were held in phosphate-buffered saline after removal of 5-aminouracil and irradiated with varying incident doses (12.5 to 100 ergs/mm^2) of 2650-A uv. In the second series of experiments, cells were irradiated in standard medium with one dose (50 ergs/mm^2) at various stages in the DNA-synthetic period (S). Immediately after irradiation, the medium was replaced with one containing ^{3}H-thymidine. Periodic samples were collected to determine the total amount of incorporated radioactive precursor in cells by the liquid scintillation counting method.[7]

Results and Conclusions. – In unirradiated cultures in which ^{3}H-thymidine was included in the medium immediately after removal of 5-aminouracil, the accumulated radioactivity reached a maximum (completion of synthesis) by the seventh hour. Within the range of uv incident dose tested, the cell's capacity to incorporate the precursor was severely impaired. Although this initial suppression of DNA synthesis was generally dose dependent, increasing the dose did not proportionally increase its effectiveness.

During the 15-hr postirradiation period, cells irradiated with doses lower than 50 ergs/mm^2 continued to incorporate the precursor to a limited extent but never to the control level. Cells irradiated with 100 ergs/mm^2 were incapable to incorporate the precursor beyond 20% of the control value. It is not certain whether this continued incorporation represents resumption of synthesis after delay, synthesis in unirradiated fractions of the population, or "recovery" from injury.

Irradiation of cells during various stages of S appears to have equally suppressed the rate of incorporation – at least during the early postirradiation periods. This observation differs from that of Dendy and Cleaver,[8] who reported in mouse L cells that the uv effect on rate of DNA synthesis

increases steadily as the DNA value for the cell increases. Experiments are under way to find the possible involvement of uv-induced thymine dimers in producing the effect on DNA synthesis reported here.

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24.10 CARCINOGEN BIOLOGY

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Introduction. – Carcinogen biology is the general term being proposed for studies of the effects of carcinogenic chemicals and control substances on widely separated types of biological systems. The name is an obvious analogy to the expression "radiobiology," which deals with radiation effects on widely separated systems; and like radiobiology, carcinogen biology includes the study of delayed effects of which the most notable is neoplasia.

Neoplasia as a delayed effect of chemical exposure has been the major end point in carcinogen biology for several decades. This end point also is the main focus of nearly all existing and proposed programs sponsored at a national level in cancer research.

The present research has been part of an attempt to determine if other parameters of chemical carcinogen exposure can be developed in a diverse group of biological materials that will give insight into the mechanisms in carcinogen biology in the same manner that many radiation effects end points have contributed greatly to understanding mechanisms in radiobiology. End points other than neoplasia include genetic (point mutations), cytogenetic, immunologic, pharmacologic, metabolic, and toxic effects of carcinogenic chemicals and related control substances. The last effect is especially interesting to us because acute toxicity of chemicals parallels acute radiation exposure at many points, and thus protection and recovery experiments involving chemical injury are immediately suggested.

Methods. – Acute toxicity for a representative member of each of five major classes of carcinogenic substances was determined using one or more routes of administration in hybrid mice. Some pathologic studies were made in addition to determination of time of death. Protection with AET was studied by giving it before the carcinogen, and bone marrow transplantation was tried after administration of the polycyclic hydrocarbon dimethylbenzanthracene (DMBA) for possible reduction in mortality.

Results. – Nearly all the representative carcinogens were extremely necrotizing at the site of injection, and systemic damage to bone marrow and lymphatic tissues was present. A peculiar two-day death syndrome was commonly seen with high doses of any carcinogen. Some evidence was found that bone marrow transplanted in DMBA-treated animals. AET alone or AET plus glutathione did not neutralize the acute toxicity of any of the chemical carcinogens investigated.

Discussion. – These studies have demonstrated a need for development of preparations of carcinogenic substances that can be administered readily to mammals and other organisms by many routes over a wide dose range. Inability to inject large doses intravenously handicapped our work in mice to a considerable extent. Presumably work with exaggerated acute effects of chemical carcinogens in mice will help yield the data needed to create a research field of carcinogen biology. This would be a logical development for support of tumor induction programs.

References

[1]Chemical Protection and Enzyme Catalysis section.

24.11 ELECTRON-MICROSCOPIC STUDY OF THE GERMINAL CENTER

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Introduction. – The germinal centers of lymphatic tissues undergo cyclic changes depending perhaps on many factors. Germinal center changes can be effected by administration of antigens resulting in recognizable stages in their development. Also x irradiation or actinomycin D effects changes in germinal centers, in a more or less reproducible manner, which depend, to some degree at least, on exposure or dosages employed. The purpose of this report is to describe the fine structure of mouse spleen germinal centers under different conditions of stress as well as that encountered in control mice with unknown stimuli.

Results and Conclusion. – Three groups of mice were used for the observations reported. The first group consisted of control animals; the second group consisted of mice which received a 1-ml suspension of 10% sheep erythrocytes intravenously five days prior to sacrifice; and a third group of animals were pretreated with actinomycin D and sheep erythrocytes two days prior to sacrifice.[1] The germinal centers are either active (groups 1 and 2) or inactive and reduced to a reticular remnant (group 3).

The most prominent feature of the germinal-center remnant following actinomycin D and sheep erythrocytes is the predominance of reticular cells with large nuclei with loose chromatin and containing usually a single round nucleolus. Many infolded cell membranes and elongated, interdigitating cell processes are present. Small alveolate vesicles are numerous in the cell processes as well as in the cytoplasm of reticular cells. Macrophages are present with varying quantities of electron-dense bodies, some of which are probably lysosomes. Extracellular collagenous fibrils are present in bundles, usually embedded in an electron-dense matrix. An occasional lymphocyte or plasma cell is encountered in thin sections of these germinal centers. Desmosomes or terminal bars are also observed, most likely between reticular cell processes.

In germinal centers of antigen-stimulated mice (group 2), there are large cells, each of which has a cytoplasm studded with ribosomes or polysomes, lymphocytes of various sizes, numerous plasma cells, and macrophages containing debris. In addition, reticular cells and extracellular collagenous fibrils as described above are present. Small cells with dense cytoplasm, some of which are phagocytic, correspond to Maximow's small, dark reticular cells as described by light microscopy. Germinal centers of control mice were similar except that fewer plasma cells were observed than in the antigen-stimulated group. Numerous small, dense bodies appeared in extensive extracellular spaces and in the cytoplasm of phagocytic reticular cells.

In addition to the possibility that lymphocytes invade the germinal centers and transform into the large cells which are considered immunologically competent, the potentiality of the small, dark reticular cells which resemble lymphocytes must be considered.

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24.12 STUDIES ON MUTATIONS INDUCED IN G_2 BY TEM[1]

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E. G. Bailiff Stella W. Perdue

Previous studies[2] have shown that x irradiation of *Paramecium aurelia* in G_2 (postduplication interphase) produces too few mutations to make studies possible. The chemical mutagen triethylenemelamine (TEM), however, produces an adequate yield of mutations in G_2. Consequently, a study of some of the properties of the mutation process initiated in G_2 was undertaken with this mutagen.

We have shown previously[3] that the yield of TEM-induced G_1 mutations, like that of x-ray-induced ones, can be considerably decreased by postirradiation treatment with streptomycin. This decrease occurs only if the streptomycin treatment is begun before the DNA synthesis immediately following the G_1 period. This and other data have led to the conclusion that many of the x-ray- and TEM-induced mutations arise from initially reparable premutational damage that is converted to final mutation only when the chromosomes replicate. The question arises as to whether mutations

induced in G_2 also arise from initially reparable damage and, if so, whether mitosis (which immediately follows G_2) or the DNA synthesis in the next cell cycle is the end point. The results showed that the yield of G_2 mutations could be at least halved by streptomycin posttreatment and that the end point for this action is after mitosis and is probably the next DNA duplication. These G_2 mutations, like G_1 ones, are to a considerable extent the result of premutational damage that produces final mutation at DNA synthesis.

The distribution of the G_2 mutations to the products of the first three postirradiation divisions was also studied. No evidence for mutations affecting both products of the first division was found. Such mutations would not have been expected because the chromosomes should be double at the time of treatment. Many of the mutations affected only one product of the second division or one product of the third division after treatment. Such fractional mutations were also found with TEM-induced G_1 mutations, but the frequency of the different classes of fractionals appears to differ for mutations induced in the two parts of the cell cycle.

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24.13 SPONTANEOUS COPOLYMERS FROM *MICROCOCCUS LYSODEIKTICUS* DNA POLYMERASE

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Micrococcus lysodeikticus DNA polymerase catalyzes synthesis of deoxyadenylate-deoxythymidylate copolymers when dATP and dTTP are incubated with the enzyme for prolonged periods in the absence of template.

Our initial assumption, based largely on base analysis, was that the products were alternating copolymers. This appeared to be partly incorrect when melting studies on primed products demonstrated two temperature transitions. A major hyperchromic effect occurred at 64.5°, and the second rise was clearly present at 73° (in SSC). The first transition corresponds to the known T_m of dAT:dAT (alternating copolymer), and the second is slightly higher than the T_m of dA:dT (homopolymer complex).

Table 24.13.1. Replication of Deoxyadenylate: Deoxythymidylate Polymers *M. Lysodeikticus* DNA Polymerase

Template	Substrate	Labeled Substrate Incorporated per 15 min (millimicromoles)
dAT:dAT	A^a	0
	T^a	0
	$A^a + T$	1.510
	$T^a + A$	1.511
dA:dT	A^a	0.858
	T^a	0.126
	$A^a + T$	1.084
	$T^a + A$	0.680

[a]Denotes labeled substrate.

Further analyses of unprimed polymers now clearly indicate that either dA : dT or dAT : dAT may arise in unprimed reactions with certain *Micrococcus lysodeikticus* DNA polymerase fractions. These two kinds of polymers are generally indistinguishable by base analysis but are readily differentiated by the melting characteristics and replication behavior. Table 24.13.1 shows the latter study and clearly indicates the requirement for both dATP and dTTP for primed synthesis of the alternating copolymers. The replication of dA : dT is quite unusual, with preferential synthesis of the dA chain and relatively independent synthesis of the two chains.

The findings are of considerable theoretical interest since they indicate that the homopolymer complex is as "normal" a spontaneous structure as the alternating copolymer. Only the alternating copolymer occurs with the most highly purified enzyme fractions, but the greater stability of dA : dT poses a paradox. The best statement we can make at this time is as follows: The more stable dA : dT helix is different in structure from dAT : dAT – perhaps dA : dT is not "Watson-Crick paired." While dAT : dAT has the x-ray diffraction pattern of DNA, dA : dT does not. Thus the most commonly occurring structure is the one that replicates easier – dAT : dAT.

24.14 DNA POLYMERASE ACTIVITY IN SYNCHRONIZED MAMMALIAN CELL CULTURES

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The enzyme DNA polymerase has been investigated in extracts of Chinese hamster cells grown in tissue culture. This enzyme is similar to the calf thymus and T2 bacteriophage–induced DNA polymerases in that it requires a single-stranded DNA primer. We have set out to answer three fundamental questions in these experiments:

1. What is the level of DNA polymerase activity as a function of the state of the cells with respect to the cell division cycle?
2. Where in the cell is the enzyme located, and does this depend on the cell division cycle?
3. Is the enzyme bound to DNA or other cell structures during the course of the cell division cycle, particularly during the period of DNA synthesis?

For measurements of total polymerase activity, cells are washed free of growth medium, suspended in 0.05 *M* Tris, pH 8, and 0.25 *M* sucrose, frozen in liquid nitrogen, and thawed. Several concentrations of this suspension are incubated for 30 min at 37° with the four deoxyribonucleoside triphosphates (one labeled with ^{14}C), 1.6×10^{-3} *M* Mg^{2+}, and heated DNA. Radioactivity incorporated is measured by collecting the acid-precipitable material on a glass-fiber disk and counting in a scintillation counter. This treatment does not disrupt the cell nucleus, as observed by light microscopy. However, mechanical homogenization or sonication does not increase the activity in the suspension.

Cells are synchronized by exposure to 5-aminouracil, which arrests the entire population early in the DNA-synthetic (S) period. When this substance is removed, the cells resume growth and proceed synchronously to cell division in 6–7 hr. Assays of polymerase activity taken at 1-hr intervals show a rise in total polymerase activity corresponding to the increase in cell protein. The specific activity of DNA polymerase (activity per milligram of protein) remains nearly constant throughout the entire division cycle, including metaphase. Experiments to determine the rate of turnover of DNA polymerase by inhibiting protein synthesis with puromycin are in progress.

The location of the DNA polymerase within the cell and its state of binding have proved to be difficult problems for which conclusive results are not yet forthcoming. The Tris-sucrose buffer results in the rupture of the cell membrane after a few hours in the cold. However, relatively little polymerase activity is obtained until the cells are frozen and thawed. Subsequent sedimentation of the nuclei and cell debris leaves most of the polymerase in the clear supernatant, while the bulk of the protein is sedimented. This suggests that the freezing and thawing treatment alters the nucleus sufficiently for the polymerase to be extracted in a soluble form.

Using a completely different approach, cells are layered on top of a sucrose gradient containing a detergent layer. The cells are lysed completely as they pass through the detergent, leaving the cytoplasmic contents behind while the heavier nuclei band at the higher sucrose density. The polymerase activity is found only in the cytoplasmic layer.

A third method of lysis, employing potassium bicarbonate at pH 9.2, completely disrupts the cell structure, producing a viscous, stringy clump that cannot be resuspended. If, after centrifugation, the supernatant is collected and the stringy precipitate washed and sonicated so that it can be resuspended, approximately one-third of the total polymerase activity is found associated with the pellet fraction, even in randomly growing cells.

In view of the above conflicting results it would appear that our lack of knowledge about the structure of the nucleus, its permeability, and the many possible ways in which cellular proteins and substructures may bind renders the extrapolation of any results on disrupted cells to intact cells meaningless. However, one may postulate certain specific interactions, carry out lysis under conditions which will not disturb these, and see whether or not they occur. We are currently refining techniques for the demonstration of possible binding between the chromatin and DNA polymerase during the period of DNA synthesis.

References

[1]Chromosome Cytology section.

24.15 HYDROXYLAMINE AS A MUTAGENIC AGENT FOR *NEUROSPORA CRASSA*

H. V. Malling

Hydroxylamine (HA) induces base-pair substitutions in phage, and the type of the genetic alterations is preferentially from guanine-cytosine (GC) to adenine-thymine (AT).[1] The chemical reaction between HA and DNA has been analyzed by Freese *et al.*,[2] who found that HA reacts only with cytosine and hydroxymethylcytosine (HMC).

HA has been shown to induce chromosome damage in mammalian cells,[3] but no previous attempt has succeeded in identifying at the molecular level the genetic alteration induced by HA in eucaryotes. If HA induces mutations in *Neurospora*, which predominantly are transitions from GC $\longrightarrow$ AT, HA would be a useful tool to characterize the mutations induced by other mutagenic agents on the molecular level.

Measurement of the reversion frequencies in the tester set[4] for study of the mutagenic action of any treatment is a much easier assay system than most forward-mutation systems. Therefore the conditions for using HA as a mutagen in *Neurospora* were developed in this tester set. It was

Table 24.15.1. Survival Percentage and the Reversion Frequencies of the Tester Set After Treatment with ICR-170, NA, EMS, and HA

Mutant Number	Survival Percentage				Reversions per 10^8 Survivors				
	NA	EMS	ICR-170	HA	SP	NA	EMS	ICR-170	HA
2-17-8	75	77	86	54	1	0[a]	0[a]	5	0[a]
2-17-23	80	95	61	62	0.3	0[a]	0[a]	0[a]	0[a]
5-4-1	88	77	68	50	0.2	0[a]	5	1938	0[a]
2-17-7	50	69	70	90	4	0[a]	5	749	0[a]
2-17-61	80	68	62	60	5	183	198	0[a]	45
2-17-155	93	99	72	62	1	79	377	8	20
2-17-18	61	71	61	64	3	182	10	91	0[a]
2-17-126	82	95	90	68	4	136	77	146	8

[a]0 means that the reversion frequency is not different from the spontaneous mutation frequency ($P \geqq 0.05$).

found that HA induces reversions in three of the four strains which revert by base-pair substitutions (Table 24.15.1). The failure of certain base-pair substitution mutants to revert with HA is easily understandable from knowledge that HA preferentially induces base-pair transitions from GC to AT. It can therefore be expected that there exist types of base-pair substitution mutants which cannot be reverted by HA. We may therefore assume that HA in *Neurospora* induces mutations preferentially by reacting with the GC pair.

It is important, however, to investigate the general mutagenicity of HA with respect to induction of chromosome breakage and induction of single base-pair changes. This information can be obtained by analyzing mutants isolated by the direct method in de Serres's dikaryon system.

In two experiments 700 HA mutants were isolated, and 250 mutants are now being analyzed with respect to their genotype and their specific reversibility after treatment with HA, NA (nitrous acid), EMS (ethyl methanesulfonate), and ICR-170 (a monofunctional acridine mustard).

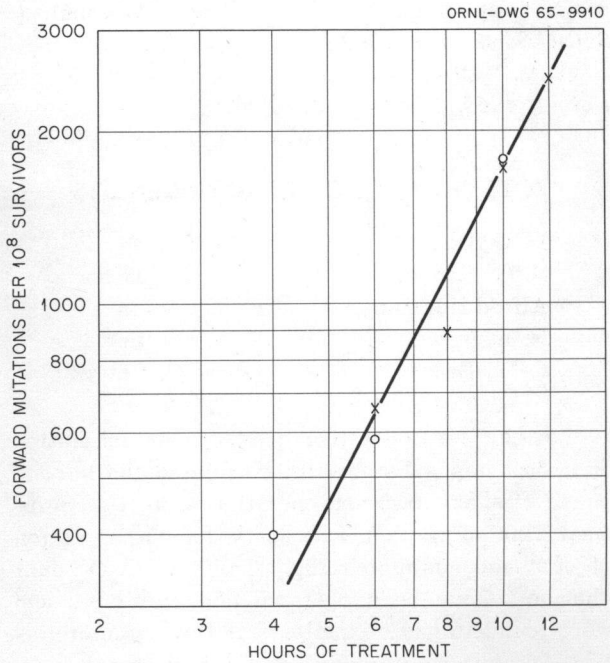

Fig. 24.15.2. Kinetics of the Induction of Forward Mutation (Purple Mutants) in a Dikaryon by Hydroxylamine. The conidia were heated in 1 *M* hydroxylamine (pH = 6.2) and 3 *M* NaCl at 25°C.

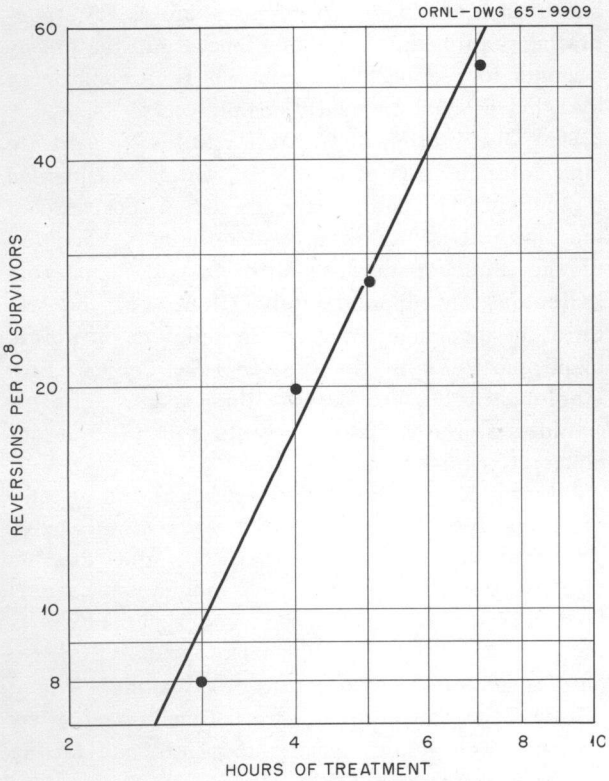

Fig. 24.15.1. Kinetics of the Induction of Reversions by Hydroxylamine in the Base-Pair Substitution Mutant 2-17-155 (Logarithmic Plot). The conidia were heated in 1 *M* hydroxylamine (pH = 6.2) and 3 *M* NaCl at 25°C.

The kinetics of induction of forward and reverse mutations by HA follows a second-order kinetics (Figs. 24.15.1 and 24.15.2), whereas induction of forward mutation by NA in the dikaryon follows a first-order kinetics and the order of induction of reversion mutations by NA varies. The second-order kinetics for induction of mutations by HA is readily explained by the fact that the reaction of HA with cytosine occurs in two steps, each step using one molecule of HA. On the basis of these results it seems most likely to assume that it is the last transformation of cytosine which induces the transition from GC to AT in the following replications.

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24.16 INHALATION CARCINOGENESIS

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The chamber operation part of the Inhalation Program[1] has substantially completed the second phase of shakedown and operation of all its equipment, including that necessary for the injection of dust and gasoline smog. The two Cr_2O_3 dust chambers have been operated and calibrated and were found to have excellent air flow characteristics. These were ascertained under normal operating conditions and with a normal load of 45 empty animal exposure cages. Operating conditions included: (1) 10 air changes/hr (10 cfm), (2) a Cr_2O_3 concentration of 25 mg per cubic meter of chamber air, (3) a 70°F temperature, and (4) a 50% relative humidity. The calibration was done gravimetrically.

The degree of aerosol homogeneity within each of the five exposure levels was determined by means of aerosol samples collected simultaneously from beneath the midpoint of the nine exposure cages. The means and 95% confidence limits reported in the following table indicate excellent homogeneity within each level.

Exposure Level	Mean Sample Concentration (mg of Cr_2O_3 per liter)	95% Confidence Level
1 (bottom)	0.021	±0.000013
2	0.026	0.000020
3	0.029	0.000026
4	0.028	0.000028
5	0.026	0.000029

The variation among exposure levels was obtained by sampling simultaneously at nine locations over the five levels, two random locations at each of four levels, and one sample at the midpoint of the middle level. No significant variation among levels was indicated. The overall mean and 95% confidence limits were 0.0263 mg/liter and 0.00005. The sampler concentrations are reported in the table below.

Sampler	Level	Sampler Concentration (mg of Cr_2O_3 per liter)
1	1	0.029
2	1	0.023
3	2	0.024
4	2	0.024
5	3	0.027
6	4	0.029
7	4	0.025
8	5	0.028
9	5	0.028

The ozone generating and sampling equipment has been installed in chambers 3 and 4. The generating equipment has been operating for nearly a month to season the chambers. It is considered that this process is nearly complete.

Size Distribution of Cr_2O_3 Particles. – Particle size determination of the Cr_2O_3 which was passed through a fluid energy mill to reduce the size of the material is continuing. Preliminary sampling of the chamber to date with cascade impactors indicates that approximately 70–80% of the material in the chamber air is in the form of single ovoid particles in the 0.5- to 1.5-μ range. Few single particles are outside this range. The remainder of the aerosol consists primarily of agglomerates larger than 3 μ.

A factor of concern in the program is the genetic homogeneity of the animals in the breeding colony. This has been verified by tail-skin-graft studies of 8-week-old inhalation-carcinogenesis-barrier-bred C57B1/6J strain mice. The types of grafts tested were isologous, homologous (BC3F1), autologous, and cross grafts from another inbred C57B1/6 strain obtained from Cumberland View Farms. The results among the male and female groups are shown in Table 24.16.1.

Results of cross grafting between male and female among the ICC C57B1/6 mice showed 100% successful grafts of female on male, while the male-on-female grafts were all rejected by two

Table 24.16.1. Skin Graft Test for Genetic Homogeneity

Strain	Sex	Number of Animals	Graft	Result
C57B1/6 ICC[a]	Male	20	Isologous	95% successful
		10	Homologous	100% rejected in two weeks
		10	Autologous	100% successful
		10	CVF male	100% successful
C57B1/6 CVF[b]	Male	18	Isologous	90% successful
		9	Homologous	100% successful
		9	Autologous	100% successful
		9	ICC male	90% successful
C57B1/6 ICC[a]	Female	16	Isologous	90% successful
		8	Homologous	100% rejected in two weeks
		8	Autologous	100% successful
		8	CVF female	100% successful
C57B1/6 CVF[b]	Female	18	Isologous	94% successful
		9	Homologous	100% rejected in two weeks
		9	Autologous	79% successful
		9	ICC female	90% successful

[a]Inhalation carcinogenesis C57B1/6 strain.
[b]Cumberland View Farms C57B1/6 strain.

months. This is expected due to the Y antigen. The results of this study indicate that the barrier-bred C57B1/6 mice being used in the inhalation carcinogenesis program are an inbred strain genetically comparable to a leading commercial strain.

A complete radiation recovery experiment has been performed in the 4-week-old barrier-bred C57B1/6J mice at a 100-r x-ray dose. These results show a 60% decrease in the white blood cells of peripheral blood by 24 hr postirradiation, with complete recovery of this parameter between 10 and 12 days postirradiation. No significant change was measured in the peripheral red cell count or hematocrit. Because of the lack of any histologic damage of this 100-r dose on the upper and lower respiratory tract, this is the reasonable radiation dose to be used in the inhalation carcinogenesis experiment.

The problem of viral infection via an aerosol route and the response of the C57B1/6J mice have been extensively examined. Chambers of two differing dimensions have been employed in these studies. As many as 100 animals may be infected simultaneously in the smaller chamber (4.5 ft^3), whereas over 600 animals may be simultaneously infected in the larger chamber (60 ft^3). The majority of the studies have been performed in the smaller chamber. Initially, the distribution pattern of an aerosol cloud was determined utilizing aerosolized polio virus in conjunction with a suitable tissue culture substrate. Using plaque-forming units per milliliter as the criterion of distribution, it was ascertained that uniform distribution was accomplished and therefore feasible.

Following the studies of distribution analysis, C57B1/6J mice were placed in cages at various positions within the 4.5-ft^3 chamber. These animals were then exposed to 1 ml of an aerosolized 10^{-1} dilution of PR8-34-60 influenza virus for 10 sec. After delivery of this concentration of virus, the animals remained in this environment for 10 min subsequent to removal for observation. Two days after viral exposure, five mice from each group at five different locations in the chamber were sacrificed. The lungs of these animals were scored, and in all cases lung consolidation ranged from a 1+ to 2+. The remaining animals were kept until death. Seventeen out of 25 animals died at day 7, and the remaining animals died on day 8. These results established both the even

distribution of the virus and its potency in the small chamber.

An LD_{50} determination was next performed, utilizing the C57B1/6J mice in the smaller (4.5 ft^3) chamber. Table 24.16.2 illustrates the results of six separate experiments utilizing over 300 5- to 6-week-old mice. One milliliter of a 10^{-1} dilution of PR8-34-60 influenza virus brought about death of 100% of the animals exposed. At a viral dilution of 10^{-2}, better than 90% of the animals exposed had an HI titer, indicative of infection. However, no animals died from this dilution of influenza virus. When the quantity of infective material was increased by either a single exposure to ½ ml of virus/ft^3 or by double exposure, the LD_{100} was raised approximately one log unit.

Table 24.16.2. Aerosol Inoculation of PR8-34-60 Influenza Virus into C57B1/6J Mice in a 4.5-ft^3 Chamber

Experiment Number	Virus Dilution	Number of Deaths/Total Number of Animals	(+) HI Titer
1	10^{-1}	12/12	N.C.
	10^{-2}	0/12	11/12
	10^{-3}	0/12	4/12
	10^{-4}	0/12	0/12
2	10^{-1}	12/12	N.C.
	10^{-2}	0/12	10/12
	10^{-3}	0/12	2/12
	10^{-4}	0/12	0/12
3	10^{-1}	10/10	N.C.
	10^{-2}	0/10	10/10
4	10^{-1}	20/20	N.C.
	10^{-2}	0/20	20/20
5	10^{-1}	8/8	N.C.
	10^{-2}	5/8	N.C.
	10^{-3}	0/8	N.C.
	10^{-4}	0/8	N.C.
6	10^{-1}	20/20	N.C.
	10^{-2}	20/20	N.C.
	10^{-3}	0/20	N.C.
	10^{-4}	0/20	N.C.
	10^{-5}	0/20	N.C.
	10^{-6}	0/20	N.C.
	10^{-7}	0/20	N.C.

Table 24.16.3. Aerosol Inoculation of PR8-34-60 Influenza Virus into C57B1/6J Mice in a 60-ft^3 Chamber

Experiment Number	Virus Dilution	Death	Lung Consolidation
1	10^{-1}	40/40	N.C.
	10^{-2}	0/40	1+ to 2+
2	10^{-1}	38/40	N.C.
	10^{-2}	0/40	1+ to 2+

Two experiments have been conducted in the 60-ft^3 chamber to test the distribution and efficiency of viral infection. In both experiments a total of 40 animals, each in groups of 5, were placed at varying positions throughout the large chamber. A similar scheme of exposure was followed as previously described, with the exception that the total of inoculum was increased to 5 ml. Results in Table 24.16.3 again demonstrate the adequacy of the aerosol method for infecting a large population of animals. The two surviving animals in the second experiment can be explained by their rather inaccessible position to the aerosol in the chamber (see Table 24.16.3).

Since all animals which are to be utilized for the virus portion of the inhalation study must be proved to be infected with the influenza virus, it was next deemed necessary to determine the optimum time at which to test the animals for the presence of HI titer and whether it would be logistically feasible to perform serological tests on 600 animals during one 24-hr period. In a series of two experiments, utilizing over 500 mice and either the intranasal or the aerosol method of viral application, the time at which HI positive sera could first be detected was determined; results are illustrated in Table 24.16.4. Similarly, it was also proved feasible to orbital bleed 450 animals during an 8-hr time period, process the blood, and perform HI determinations in another 6 hr (see Table 24.16.4).

Due to the possibility of oncogenic viruses being present in certain chick embryos, it was desirable to obtain a resistance-inducing-factor-(RIF)-free influenza virus. The A_2NS (RIF free) influenza virus strain had only been adapted to growth in embryonated chick eggs when first received from Wyeth Laboratories. During the first 30 passages (at two-day intervals) of this agent in C57B1/6J mice, the highest HA titer attained was 1:16 with an LD_{50} of 1.5. Such a low LD_{50} was not acceptable for our purposes, since three to four log units of virus is lost by dilution when animals are infected by the aerosol method. Since several biochemical methods of enhancing the adaptation of the virus proved unsatisfactory, it was decided to utilize younger mice in order to more efficiently adapt the A_2NS virus. After 29 alternate day passages of the agent in 3-week-old C57B1/6J mice, the HA titer is now 1:64, and the LD_{50} is 2.8. Since we are primarily interested in infecting the animal without bringing about death, this degree of adaptation may prove adequate for our purposes. One aerosol experiment has been performed, and although no animals died from exposure to the A_2NS agent, lung consolidation ranged from 1+ to 3+ at 10^{-1} dilution of the virus and +/– at the 10^{-2} dilution.

Since half the C57B1/6J mice are to be exposed to x-ray irradiation prior to viral exposure, a series of experiments are currently in progress to determine whether 100 r of x ray alters the above mentioned host's susceptibility to influenza infections and at what time interval after exposure to x ray the mice respond in a "normal" fashion to viral infection. Unless x irradiation drastically alters the infectivity response, we are at this time prepared to proceed with the viral portion of the inhalation project. In the event that there is dramatic interaction between radiation and viral exposure it is feasible to reduce any additive lethal effects by reducing the titer of viral exposure encountered by the C57B1/6J mice.

Table 24.16.4. Onset of HI Titer After Infection with PR8-34-60 Influenza Virus

Experiment Number	Day After Infection	Infective Virus Titer	Percent HI(+)
1[a]	1–6	10^{-1}–10^{-6}	(–)
	7	10^{-1}	50
	7	10^{-2}	0
	9	10^{-2}	50
	11	10^{-2}	100
2[b]	1–5	10^{-1}–10^{-7}	(–)
	6	10^{-1}	100
	6	10^{-2}	75
	6	10^{-3}	75
	6	10^{-4}	100
	6	10^{-5}	50
	6	10^{-6}	25
	7	10^{-1}	100
	7	10^{-2}	100
	7	10^{-3}	100
	7	10^{-4}	100
	7	10^{-5}	75
	7	10^{-6}	66
	7	10^{-7}	75

[a]Aerosol inoculum $\frac{1}{2}$ ml/ft^3.

[b]Double exposure to $\frac{1}{2}$ ml/ft^3 at 10-min interval.

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24.17 EFFECT OF ANTIGEN DOSE ON LYMPHATIC TISSUE GERMINAL CENTER CHANGES

M. G. Hanna, Jr. C. J. Wust[1]
C. C Congdon[2]

Three consistent histologic findings during early intervals of the spleen lymphatic tissue reaction to an antigenic stimulation of 1 ml of a 10% sheep erythrocyte (SRBC) suspension have been reported.[3] The first change is germinal center loss or dissociation; the second is the occurrence of large pyronin-staining cells throughout the lymphatic nodule and splenic red pulp. The presence and general significance of these pyronin-staining cells for reaction to antigenic material have been recognized for some time, and these cells are thought by many investigators to be the source of plasma cells and other immunologic phenomena; the third change is germinal center recovery with hyperplasia beginning two days after antigen injection.

Autoradiographic and histologic studies have suggested that cells of spleen white pulp germinal

centers can be stimulated either directly or indirectly by antigen and that these stimulated cells emigrate from the center to other areas of the spleen. This response ultimately results in what has been termed the dissociative growth of germinal center cells. These studies have also suggested that on a dynamic and morphologic basis a correlation exists between germinal center cells and the large pyronin-staining cells characteristically seen in lymphatic tissue during the early intervals of the immune response.

The purpose of this study was to investigate the effect of antigen dose on spleen germinal center changes and correlate this with the production of specific serum antibody during the first 30 days after primary antigenic stimulation.

Materials and Methods. – Male $C3BF_1$ mice were randomly divided into six groups of 60 animals each. Animals in five of these groups were injected intravenously with 1 ml of 0.01, 0.1, 1.0, 10.0, and 25% sheep erythrocyte suspensions respectively. The animals in group 6 were non-treated controls. Five mice from each group were killed by severing the aortic arch at 2 hr and 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, and 30 days after antigen. Blood collected from each mouse was used for serial hemagglutination studies, while spleen collected from each mouse was prepared for histology.

For the determinations of the surface areas of the white pulp germinal centers in each longitudinal spleen section, the image of the spleen section was microprojected at a magnification of 120×. The outlines of the germinal centers were traced on tracing paper, and the respective areas were measured with a compensating polar planimeter.

Results and Conclusion. – The results of the present study indicate that the dissociation or loss of germinal center during the first 24 hr after SRBC antigen is dose dependent, with a direct relation existing between germinal center loss or atrophy and increasing antigen dose, and also that atrophy is only achieved at higher antigen doses which are capable of drastically altering the flux of cells in the centers. In this study an antigen dose to which the germinal centers respond by hyperplasia alone appears to be approached with the 0.01% SRBC suspension.

Several investigations concerned with changes in lymphoid tissue during antibody formation have failed to detect the germinal center loss or atrophy while describing the hyperplasia. These studies have mainly used low doses of soluble antigens. Based on the present work this would imply that the antigen type and dose were incapable of altering the flux of cells in the centers to the point that there was a resulting atrophy.

There is an interesting correlation between the degree of germinal center hyperplasia and serum hemagglutinin production. At the lower antigen doses (0.01 and 0.1% SRBC), the degree and duration of the hyperplasia are proportional to the serum hemagglutinin titer. At greater antigen doses, where high titers are maintained to 30 days, the hyperplasia is also of a sustained nature. The role of the hyperplastic germinal centers in the immune response and their relationship to the serum hemagglutinin are questions of great importance.

The correlation of the germinal center changes with serum hemagglutinin production and the dose dependency of the center changes seem to provide another parameter for measurement of the immune system. The measurement of germinal center changes would be a reflection of the proliferation and differentiation of immunogenically competent cells and might be used in situations where high serum antibody titers are not available.

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24.18 EFFECT OF ANTIGEN DOSE ON TYPE OF SPECIFIC ANTIBODY PRODUCED DURING VARIOUS INTERVALS OF THE PRIMARY IMMUNE RESPONSE

M. G. Hanna, Jr. W. D. Fisher[1]

We have reported a correlation between serum hemagglutinin production and splenic germinal center changes during the first 30 days after primary injection of antigen, with doses ranging from 0.01 to 25% sheep erythrocyte suspensions.[2] A study was also performed to determine what effect antigen dose has on the type of antibody produced and how this correlated with the rate and amount of antibody which could be serially

measured during the first 30 days after these various antigen doses were administered. Measurements were made to determine the amounts of "light" (primarily 7S) and "heavy" (primarily 19S) antibodies. Separation of the immunoglobulins from serum was achieved by zonal centrifugation.

Materials and Methods. – Male BC3F1 mice were randomly separated into six groups of 30 animals. The first five groups were intravenously injected respectively with 1 ml of 0.01, 0.1, 1.0, 10.0, and 25% sheep erythrocyte (SRBC) suspensions. The sixth group was used as a noninjected control. Ten mice from each group were killed by severing the aortic arch on days 6, 12, and 30 after antigen. At each interval the blood from each group of 10 mice was pooled, and approximately 4 to 5 ml of serum was separated by centrifugation.

The immunoglobulin from approximately 5 ml of pooled serum was separated in the B-IV zonal rotor on a 1-liter 10 to 20% sucrose gradient, linear with radius and containing Miller-Goldner buffer (0.2 ionic strength), by centrifugation to 3×10^6 $\omega^2 t$ (approximately $6\frac{1}{2}$ hr at 40,000 rpm). The gradient was collected in cuts of 40 ml. Samples from each cut were serially titrated against 1% SRBC for specific hemagglutinin.

Duplicate samples were also titrated in the presence of 0.1 *M* 2-mercaptoethanol (2ME). This was done to test the relative sensitivity of the light and heavy immunoglobulins, based on early reports which indicate that 19S is 2ME sensitive while 7S is not.

Results and Conclusion. – Based on optical density measurement of the gradient, the light immunoglobulin was obtained in cuts 8 through 22, while the heavy immunoglobulin was obtained in cuts 22 through 26. In all control groups no specific antibody against SRBC was measured in these fractions.

The results of these studies are shown in Table 24.18.1. The proportion of heavy to light immunoglobulins at day 6 after antigen is dose dependent, with an increasing proportion of light being measured at day 6 with increasing antigen dose. At the 12- and 30-day intervals the measurement shows primarily light immunoglobulins, indicating a shift in the type of antibody being produced at different intervals of the immune response.

The measurements with 2ME indicate that a part of the light immunoglobulin fraction is sensitive to it. Thus, if 7S immunoglobulin is truly insensitive to this agent, not all the immunoglobulin in the light fraction obtained from the zonal centrifuge is 7S.

Further studies are contemplated to refine and use this system to measure the types of immunoglobulins produced in various immune reactions.

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Table 24.18.1. Percent Heavy and Light Hemagglutinin

Dose	Days After Antigen Injection					
	6		12		30	
	Percent Heavy	Percent Light	Percent Heavy	Percent Light	Percent Heavy	Percent Light
0.01% SRBC	100					
0.1% SRBC	74	26	37	63		
1.0% SRBC	55	45	7	93	6	94
10.0% SRBC	57	43	22	78	4	96
25.0% SRBC	65	35	24	76	5	95

24.19 PHYSIOLOGY OF THE VIRUS-HOST INTERACTION

Alfred Hellman D. H. Martin
J. G. Farrelly

In our efforts to further delineate factors associated with viral infection as reflected by the physiological status of the host, two parameters are currently under investigation.

If viral infection is to occur, bringing about either recognizable cellular destruction or, as in the case of oncogenic virus, possible alterations in the host cell genome, the virus must be able to enter the cell. Dimethylsulfoxide (DMSO), a substance recognized for its permeation qualities, might prove an ideal vehicle for the enhancement of viral penetration into the host cell. In our initial studies it was observed that DMSO enters the cell rapidly and, if in contact with a growing cell population for more than 24 hr at concentrations of 4% or greater, reduces cellular viability. Concentrations of less than 4% bring about no change in cellular viability as determined by the erythrosin B dye exclusion technique. It was further noted that DMSO influences the synthesis of viruses that are genetically distinguishable but not antigenically differentiated. Preliminary observations indicate that DMSO might serve as another genetic marker for the differentiation between attenuated and virulent polio viruses, since the attenuated strain demonstrates a 200% greater inhibition of viral synthesis than the virulent strain with low concentrations of DMSO.

We are currently studying other parameters possibly associated with the aforementioned phenomenon, such as rate of virus adsorption in treated cells and the competency of treated cells to synthesize new virus, as well as the rate of viral synthesis as influenced by DMSO. The significance of such a study as related to viral oncogenesis is associated with the question posed by genetic alterations of the host upon viral infection. Therefore, chemical therapy may bring about selective inhibition of antigenically related viral agents and negate the mutagenic factors of certain viruses.

In a related study the influence of the ionic environment on viral synthesis is being investigated. One finds homeostasis in an in vivo situation, whereas changes in ionic environment do occur in an in vitro cellular system. The influence of a constant ionic environment in an in vitro system is currently under investigation in efforts to determine the validity of existent data obtained in the whole animal as related to viral synthesis performed in an in vitro state. Thus far we have been successful in maintaining an in vitro cell population at a constant pH (within 0.01 pH unit) for an indefinite time. The influence of constant vs continually changing pH, at and around the physiological level, on the cell population is being investigated utilizing the parameters of protein synthesis, amino acid utilization, nucleic acid synthesis, cellular generation time, cellular viability, and cell size distribution. Subsequently we will determine the influence of this environmental factor on viral synthesis.

Hydrogen ion concentration, as well as other environmental factors associated with viral penetration and synthesis, is intimately related to the viral oncogenic process, particularly as associated with those studies comparing in vivo and in vitro viral initiated mutagenesis, and in the animal's susceptibility to potential viral oncogenic agents.

In yet another host-environment related problem, we are reinvestigating the influence of various doses of irradiation on viral replication and pathogenesis. This study will determine the influence of animal age, radiation dose, and viral dose relationship on pathogenicity; comparative studies will be performed in an in vitro cellular system. Conventional virological parameters will be utilized to evaluate results obtained in both experimental situations. The influence of radiation on host enhancement of viral susceptibility as influenced by viremia will similarly be investigated. Once more, the casual relationship of viruses to some of the as yet unexplained phenomena of radiation death will be studied utilizing the myxoviruses and enteroviruses as models.

24.20 SOME FACTORS THAT AFFECT BENZO(A)PYRENE UPTAKE BY BACTERIA

B. G. Moore A. P. Harrison, Jr.

Some strains of *Escherichia coli* and other bacteria (Table 24.20.1) bind 3,4-benzo(a)pyrene when cultivated in serum-synthetic medium (SSM).

Table 24.20.1. Benzo(a)pyrene Uptake by Various Enterobacteriaceae[a]

Culture	Micrograms of Bound Benzo(a)pyrene/Cell
	$\times 10^{-10}$
E. coli 1712	3.0
Salmonella typhimurium 698	1.9
E. coli Ma	1.3[b]
E. coli C_{T-}	0.80
E. coli 1062-62	0.50
E. coli 1062-62 (mucoid)	0.70
E. coli B	0.44
E. coli Bi316-42	0.30
E. coli Bi316-42 (mucoid)	0.34
Aerobacter aerogenes t	0.30
E. coli 1529-63	0.25
E. coli 1529-63 (mucoid)	0.31
E. coli K12 (×323)	0.20
E. coli 15	0.10
E. coli Ma (autoclaved)[c]	0.05

[a]Cells were cultivated on the shaker for 6 hr at a cell concentration of 10^9/ml in SB containing 10 μg/ml chloramphenicol and 25 μg/ml ^{3}H-benzo(a)pyrene at 10^6 counts min^{-1} ml^{-1}.

[b]The mean from five experiments where bound benzo(a)pyrene was 1.1, 1.5, 1.3, 1.2, and 1.2 $\times 10^{-10}$ μg/cell.

[c]For 10 min at 15 lb (121°C).

Bound benzo(a)pyrene is defined as that benzo(a)pyrene which remains associated with the bacterial cells after three washings with fresh SSM. (Further washings remove negligible additional hydrocarbon.) Uptake is defined as the ability of cells to acquire bound benzo(a)pyrene. It is measured by employing tritium-labeled hydrocarbon. Critical experiments have employed a ^{14}C label to ensure that spurious tritium exchange did not occur. This report describes several of the factors which affect uptake. Pertinent data are presented in three figures. Additional data are presented elsewhere.[1]

Figure 24.20.1 compares uptake by nongrowing cells with that by an actively growing culture which attains the same cell titer, and demonstrates that the presence of chloramphenicol and the absence of leucine do not inhibit uptake. Under these circumstances there is no measurable increase in protein (Folin tests). Thus, we conclude that active protein synthesis is not necessary for uptake to occur. The figure also illustrates that absence of glucose diminishes uptake but slightly. (However, cells previously starved 24 hr require glucose for maximum uptake.) Figure 24.20.2 illustrates that heating the inoculum at temperatures slightly in excess of growth temperature – maximum growth temperature for *E. coli* lies between 45 and 50°C – enhances subsequent benzo(a)pyrene uptake. However, autoclaving the cells for 5 min diminishes uptake but does not eliminate it entirely. Thus we conclude that some uptake must occur in absence of enzymatic activity. Figure 24.20.3 shows uptake occurring simultaneously with thymineless death. Folin tests indicated protein increase in proportion to mass. Thus, based on unit mass (protein or nephelometric), uptake is sevenfold greater in the thymineless suspension. The same rationale applies in the case of the uptake per unit mass in suspensions treated with penicillin, especially if the cells are prevented from lysing by incorporating 20% sucrose which permits formation of protoplasts.

The above data stress that uptake of the hydrocarbon is affected by the physiological state of the cells. Continuing study of factors that influence hydrocarbon uptake, hopefully, will suggest interactions between cellular functions and uptake of carcinogenic hydrocarbons.

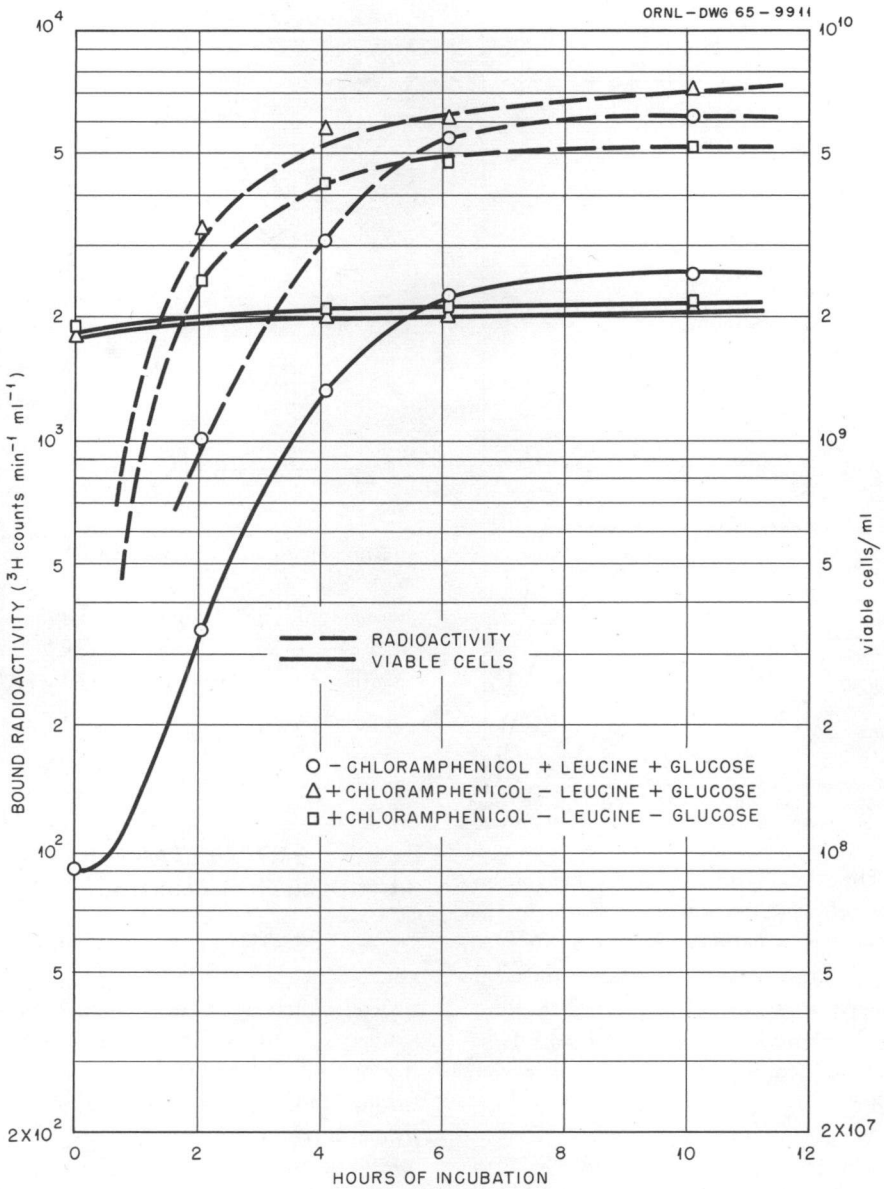

Fig. 24.20.1. Comparison of Uptake by Nongrowing and Actively Growing *E. coli* Ma Cells. The three suspensions were in serum-synthetic medium containing 25 μg/ml ^{3}H-benzo(a)pyrene at 6×10^5 counts min^{-1} ml^{-1}.

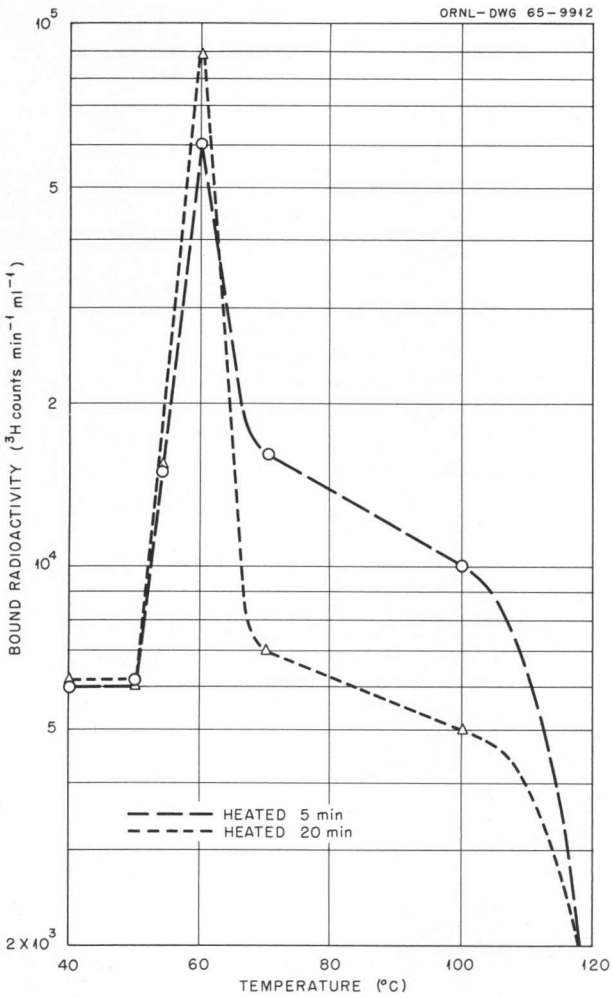

Fig. 24.20.2. Effect of "Heat Shock" on Benzo(a)pyrene Uptake by *E. coli* Ma. Cells from an overnight agar culture were suspended in buffer at a titer of 10^{11}/ml, were heated as indicated, then were diluted 10^{-2} into 38°C serum-synthetic medium lacking leucine but containing 10 μg/ml chloramphenicol and 25 μg/ml ^{3}H-benzo(a)pyrene at 10^6 counts min^{-1} ml^{-1}, and were incubated for 22 hr.

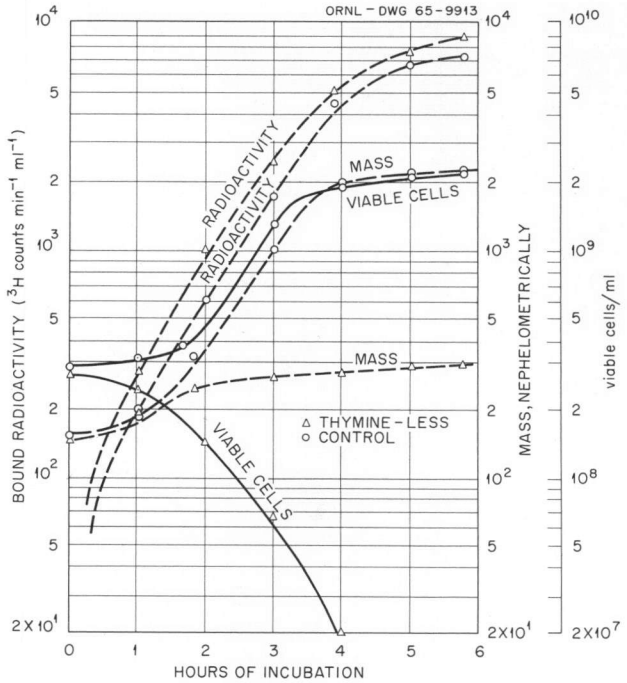

Fig. 24.20.3. Comparison of Uptake by *E. coli* Ma With and Without Thymine. Cells were incubated in serum-synthetic medium containing 25 μg/ml ^{3}H-benzo(a)pyrene at 10^6 counts min^{-1} ml^{-1}. Mass was monitored nephelometrically against Coleman standards.

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24.21 METABOLIC STUDIES ON 1-(4-DIMETHYLAMINOBENZYLIDENE)-INDENE

C. T. Bahner D. G. Doherty

Radiomimetic, carcinogenic, and antitumor effects of 4-(4-aminostyryl)-quinolines have been reported. Recently, a series of 1-(4-aminobenzylidene)-indenes have been prepared, and some of them have been reported to be as active against Walker 256 tumors in rats as the aminostyrylquinolines but much less toxic to the animals. The metabolism of these indenes, which may contribute to their reduced toxicity, is not presently known. As a step toward understanding the effects of these compounds, ^{14}C-labeled 1-(4-dimethylaminobenzylidene)-indene has been prepared, and experiments are currently in progress

on the fate of the compound when administered by the oral, subcutaneous, and intraperitoneal routes.

Ultracentrifuge Development Program

24.22 STUDIES OF NATIVE GLYCOGEN ISOLATED FROM SYNCHRONIZED *TETRAHYMENA PYRIFORMIS* (HSM)

A. A. Barber[1] W. W. Harris[2]
G. M. Padilla[3]

Introduction. – Most of the physical properties of glycogen, except molecular weight, are quite similar, regardless of its source.[4] For example, glycogen isolated from *Tetrahymena pyriformis* by hot KOH extraction is similar to that isolated from mammalian liver and muscle by similar extraction in all properties examined except for its higher molecular weight.[5] The hot KOH extraction procedure commonly used for preparing glycogen degrades the native material into units of much smaller size and correspondingly lower molecular weights.[6] Mild isolation procedures are therefore necessary for the studies of molecular size and sedimentation characteristics of native glycogen. Two such procedures have been recently reported.[7,8] Polydisperse liver glycogen isolated by combined rate-zonal and isopycnic centrifugation has particle sizes up to 200 mμ, with sedimentation coefficients up to approximately 4000, indicating molecular weights much higher than the KOH-extracted material.[8] Similar sizes and sedimentation coefficients for liver glycogen have been reported from several laboratories. It is not known if the native glycogen of *Tetrahymena* occurs in these larger sizes since its reported molecular weight was obtained from KOH-extracted material. Zonal centrifugation in cesium chloride gradients was carried out to compare the morphology and sedimentation properties of *Tetrahymena* glycogen with that isolated from rat liver by the same method. In addition, native and KOH-extracted glycogen were compared morphologically.

Results and Discussion. – Native glycogen was isolated from *Tetrahymena pyriformis* (HSM) by isopycnic centrifugation in cesium chloride density gradients. A density of 1.62 to 1.65 was isopycnic for glycogen. Most of the banded glycogen existed as 35 to 40 mμ particles which had a sedimentation coefficient of 214. These particles were composed of aggregates of 2- to 3-mμ spherical particles. Extraction of glycogen with hot alkali reduced the sedimentation coefficient of native glycogen from 214 to 64.7 and the particle diameter from approximately 40 to 20 mμ and smaller. Cell division was synchronized by a repetitive 12-hr temperature cycle, and glycogen was measured at several times during the cell cycle. The temperature cycle consisted of 9.4 hr at 12°C and 2.5 hr at 27°C. Approximately 90% of the cells divided during the last 1.5 hr of the warm period. The carbohydrate/protein ratio of cells at the end of the cold period was 0.27 and was reduced slightly during the warm period. Glucose was incorporated into glycogen during both periods, although the rate of incorporation was greater during the warm period. No preferential incorporation on the basis of particle size was noted. Incorporation was measured in both native and KOH-extracted glycogen.

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24.23 LIPID PEROXIDATION IN RAT TISSUE HOMOGENATES: INTERACTION OF IRON AND ASCORBIC ACID AS THE NORMAL CATALYTIC MECHANISM

A. A. Barber[1]

Introduction. – The amount of lipid peroxidation in aerobically incubated tissue homogenates depends on the tissue type and concentration,[2] the

buffer concentration, and the pH. Certain aspects of this reaction have been extensively studied using homogenates,[3] mitochondria,[4] microsomes,[2] and pure unsaturated lipids.[5] Studies using a wide variety of exogenous materials have demonstrated both an enzymic[6] and a nonenzymic[4] lipid peroxidation in microsomes and mitochondria. Iron acts as the catalyst in both the nonenzymic[4] and enzymic[7] reactions. Extensive in vitro studies have led to the establishment of an iron–ascorbic acid catalytic mechanism for nonenzymic lipid peroxidation.[8] However, the quantitative measurements of tissue iron and ascorbic acid, needed to establish their role as the normal catalytic mechanism present in homogenates, have not been carried out. This mechanism has been inferred from the many experiments using exogenous materials. The amounts of iron and ascorbic acid present in the catalytically active homogenate fractions of liver, brain, testis, and kidney, as well as the amounts of each required for the observed lipid peroxidation, are reported in this study.

Results and Discussion. – In the following paragraphs a discussion of the results is presented.

1. Iron and ascorbic acid are present in appropriate quantities in rat liver, brain, testis, and kidney fractions to explain lipid peroxidation measured in aerobically incubated homogenates of these tissues.

2. The necessity for ascorbic acid is indicated by the following: ascorbic acid occurs only in the dialyzable fraction of the soluble phase, and no peroxidation occurs in the absence of this fraction; catalysis by this dialyzable fraction is proportional to its ascorbic acid content; and the pH effect on peroxidation is similar for both ascorbic acid and soluble-phase catalysis.

3. The necessity for iron is indicated by colorimetric measurements of total microsomal iron, by EDTA inhibition studies, and by measurements of the pH effects on lipid peroxidation. These findings substantiate previously reported studies on the effects of divalent cations on lipid peroxidation.

4. Differences in the amount of peroxidation occurring in microsomes from different tissues are not related to the total iron content. We presume these differences to be related to differences in their content of specific unsaturated lipids.

5. The inhibition of lipid peroxidation noted at high tissue concentrations is due to specific inhibitors associated with the protein fraction of the soluble phase. Inhibitory activity was strongest in testis and weakest in brain soluble phases.

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24.24 ISOLATION OF MYXOVIRUS BY CONTINUOUS-FLOW CENTRIFUGATION

G. B. Cline

Introduction. – A continuous-flow centrifugation technique has been developed which permits the isolation, concentration, and purification of a relatively unstable infectious myxovirus, respiratory syncytial (RS) virus (Long strain), from large volumes of tissue culture fluid. This technique utilizes the B-IX zonal rotor with a sucrose density gradient to trap the virus and continuously band it in an isodense zone. The infectious virus is recovered from the rotor by displacement of the density gradient with a more dense solution. This isolation technique does not reduce infectivity appreciably since about 95% of the starting infectious units can be found in the gradient. Between 80 and 90% of the total starting virus is recovered in a single zone. Additional purification and concentration of the recovered virus can be obtained by a second isopycnic banding in potassium citrate in the angle-head rotor. Sufficient virus has been obtained by this technique to begin studies of the morphology of the virus and ways to disrupt it into antigenically

active subunits suitable for use as a vaccine material.

Results and Discussion. – Myxoviruses, in general, lose infectivity when concentrated by conventional centrifugation techniques or by filtration.[1] This problem of loss of infectivity has been circumvented with the B-IX rotor, which traps the virus particle from the flow-through stream and sediments it to its isopycnic zone in a density gradient. The trapped and banded virus is recovered from the rotor by displacement of the loaded gradient. Figure 24.24.1 shows the results of a typical RS run in the B-IX rotor. The infectivity zone[2] is banded at about 40% sucrose, as well as a large amount of complement-fixing (CF) antigen. The smaller zone of CF antigen centered in cuts 1 and 2 consists of viral subunits which have not yet banded in an isodense zone. Better than 80% of the starting infectious virus in this experiment (2400-ml volume) was recovered in 40 ml of gradient. More recent experiments using 10-liter batches of harvest fluid result in a virus zone of about 80 ml containing up to $10^{8.7}$ infectious units per milliliter at the peak.

Cleanout of the infectious RS particles from the flow-through stream closely follows the cleanout as theoretically predicted.[3] Table 24.24.1 shows a set of cleanout data as determined by infectivity assay of samples of the effluent stream. The cleanout efficiency ranges from 99.66% at 4 liters/hr to 99.99% at 1.2 liters/hr. This high cleanout efficiency is advantageous for RS virus or any other virus which titers low in cell cultures.

The concentration of RS virus obtained by continuous-flow centrifugation in the B-IX zonal rotor is sufficient for studies on selective disruption of the virus into subunits as well as on the intact virus itself. Electron-microscopic and biophysical studies on purified preparations of RS virus and viral subunits are now in progress.

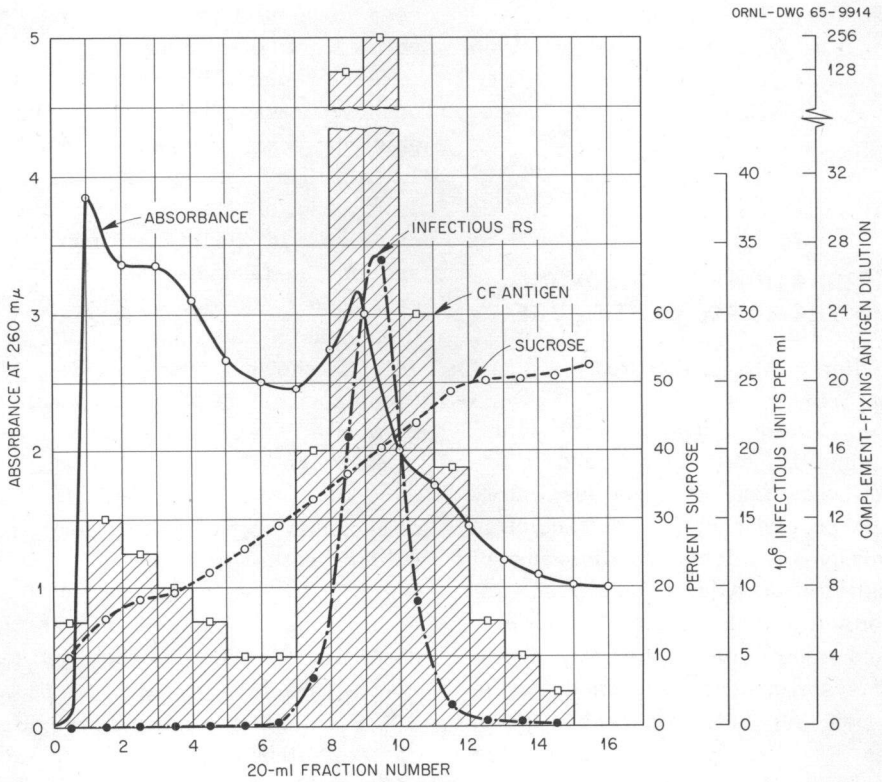

Fig. 24.24.1. Optical Density Profile Showing Infectious Respiratory Syncytial Virus Banded Isopycnically in 40% Sucrose in the Continuous-Flow B-IX Rotor.

Table 24.24.1. Cleanout of RS Virus by B-IX Zonal Rotor at 40,000 rpm

Flow Rate (liters/hr)	Infectious Units per Milliliter in Effluent	Cleanout (%)
None	3×10^6	
4.15	1×10^4	99.667
4.2	4×10^3	99.987
3.2	4×10^3	99.987
3.2	1×10^4	99.667
2.2	4.5×10^2	99.985
1.2	3×10^2	99.99

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[1]C. R. Bennett, Jr., and D. Hamre, *J. Infect. Diseases* **110**, 8–16 (1962).

[2]RS virus is grown and assayed by Laboratory of Infectious Diseases, NIAID, NIH.

[3]A. Berman, *J. Natl. Cancer Inst.*, suppl., 1965 (in preparation).

24.25 FRACTIONATION OF ADENOVIRUS ANTIGENS IN THE ZONAL CENTRIFUGE

G. B. Cline L. H. Elrod[1]

Introduction. – Certain types of adenovirus, namely, 3, 4, 7, and 12, have been found to account for a large percentage of upper respiratory disease in certain populations.[2] In collaboration with the Laboratory of Infectious Diseases of the National Institute of Allergy and Infectious Diseases in efforts to develop effective vaccines against these and other viruses, recently developed zonal-rotor systems have been used for the partial purification of antigenic subunits of adenovirus types 3, 4, and 7. The adenoviruses used in studies reported here were grown, concentrated by filtration, and disrupted into subunits elsewhere[3] prior to being analyzed in the zonal ultracentrifuge. A new zonal rotor, B-XI, which was designed primarily for isopycnic separations lasting several days, was used for these studies. Infectivity and antigen distributions have not been completed to date, so only optical density profiles of the zonal runs will be presented.

Results and Discussion. – Alkaline-disrupted adenovirus was added to potassium citrate density gradients which ranged in density from 1.175 to 1.275. The sample material was added to the 1.275 end of the gradient to ensure that free nucleic acid would sediment to the rotor wall and not contaminate the protein fractions. The capsomeres and other protein components floated to a density ranging from 1.22 to 1.24. Figure 24.25.1 shows an optical density profile of an adeno T-3 run in which 13 ml of a 100× concentrate was used as a starting sample. All the complement-fixing (CF) antigen was found to be coincident with the composite peak. The three components to the peak are believed to be comparable to the A, B, and C antigens described by Pereira[4] for type 5 adeno, but the immunological tests of zonal fractions 1, 2, and 3 have not been completed.[3] The 280/260 mμ absorbance ratio ranges from 1.15 in fraction 1 to 1.215 in fraction 3. Electron microscopy indicates the presence of the "ribbon" antigen (type-specific antigen) and capsomeres (group-specific antigen). Some undissociated virus or reaggregated virus-like

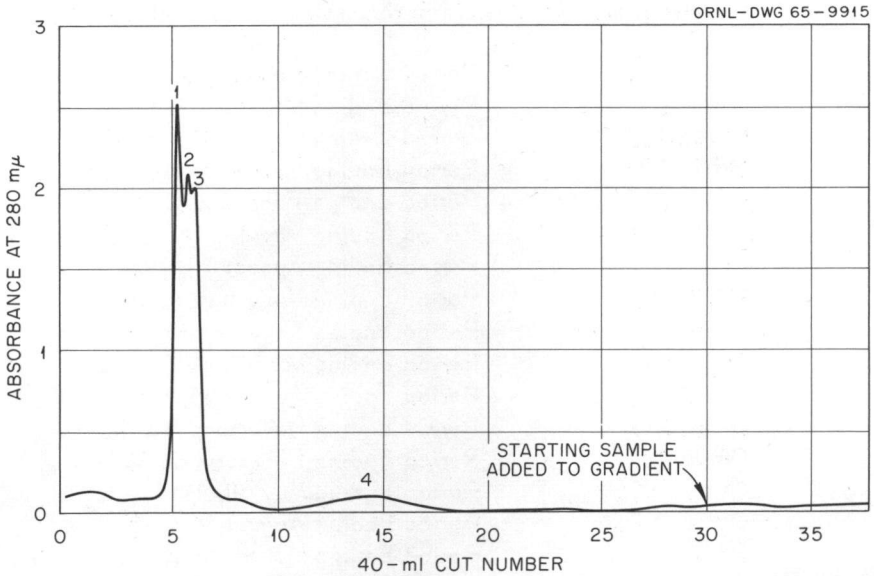

Fig. 24.25.1. Optical Density Profile Showing the Isopycnic Separation of Four Components of Alkaline-Dissociated Adenovirus Type 3 NB-XI in the B-XI Zonal Rotor.

particles have been found scattered throughout the entire density gradient as background material.

Studies are continuing in an effort to design density gradients which will give better separation of the various optical density zones and permit large-scale separation of the viral antigens from cell-associated antigens and infectious virus.

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[2]J. A. Bell, P. R. Wallace, and Leon Rosen, *Am. J. Public Health* **52**, 902 (1962).

[3]Wyeth Pharmaceutical Co., Marietta, Pa.

[4]H. G. Pereira, *Nature* **186**, 571–72 (1960).

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