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SEMIANNUAL PROGRESS REPORT

FOR PERIOD ENDING JULY 31, 1966

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BIOLOGY DIVISION
SEMIANNUAL PROGRESS REPORT
For Period Ending July 31, 1966

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NOVEMBER 1966

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22.29 Biocompatibility Tests of *Habrobracon* Under Space-Flight Conditions in the Flight Vehicle – **R. H. Smith, M. B. Baird, R. C. von Borstel, Margaret L. Haye, R. L. Amy, Ellen S. Sirota** 206

Publications and Lectures

PUBLICATIONS

During this report period (February 1, 1966–July 31, 1966), 411 papers, abstracts, and book reviews by members of the Division have been published or are in press. This total compares with 435 for the August 1, 1965–January 31, 1966, reporting period and with 343 for the February 16–July 31, 1965, period. Listed below are the 88 published papers, reports, and books edited by Division members for the current period. The 70 published abstracts, 4 published book reviews, and 249 items in press are not listed.

The proceedings of the symposium *Differentiation and Growth of Hemoglobin- and Immunoglobulin-Synthesizing Cells,* held in April 1966 at Gatlinburg, were published as a supplement to the June 1966 *Journal of Cellular Physiology.*

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Adler, H. I.	The genetic control of radiation sensitivity in microorganisms	Pp. 167–91 in *Advances in Radiation Biology,* vol. 2 (ed. by L. G. Augenstein, Ronald Mason, and Max Zelle), Academic, New York and London, 1966
Adler, H. I., W. D. Fisher, Alice A. Hardigree, and G. E. Stapleton	Repair of radiation-induced damage to the cell division mechanism of *Escherichia coli*	*J. Bacteriol.* **91**, 737–42 (1966)
Albright, J. F., and Takashi Makinodan	Growth and senescence of antibody-forming cells	*J. Cell. Physiol.* (suppl. 1) **67**, 185–206 (1966)
Allen, R. C., and Dorothy J. Moore	Sex-associated quantitative differences in the plasma esterases of inbred mice	*Endocrinology* **78**, 655–58 (1966)
Axelrod, D. E., and H. I. Adler	Episomal control of bacterial response to radiation	*Microbial Genet. Bull.* **22**, 12 (1966)
Barber, A. A.	Lipid peroxidation in rat tissue homogenates: interaction of iron and ascorbic acid as the normal catalytic mechanism	*Lipids* **1**, 146–51 (1966)
Barnett, W. E., and J. L. Epler	Fractionation and specificities of two aspartyl–ribonucleic acid and two phenylalanyl–ribonucleic acid synthetases	*Proc. Natl. Acad. Sci. U.S.* **55**, 184–89 (1966)
Bell, Sandra, and Sheldon Wolff	Effects of FUdR and thymidine on incorporation of deoxycytidine into DNA of *Vicia faba*	*Exptl. Cell Res.* **42**, 408–12 (1966)
Bender, M. A	Methods in human cytogenetics	*Arquiv. Brasil. Endocrinol. Metab.* **14**, 47–72 (1965)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Brown, R. A.	Effect of solution composition upon poly U	*Arch. Biochem. Biophys.* **115**, 102–7 (1966)
Brown, R. A., Takashi Makinodan, and J. F. Albright	Significance of a single-hit event in the initiation of antibody response	*Nature* **210**, 1383–84 (1966)
Cameron, I. L.	A periodicity of tritiated-thymidine incorporation into cytoplasmic deoxyribonucleic acid during the cell cycle of *Tetrahymena pyriformis*	*Nature* **209**, 630–31 (1966)
Cameron, I. L., D. G. Gosslee, and Christoph Pilgrim	The spatial distribution of dividing and DNA-synthesizing cells in mouse epithelium	*J. Cell. Comp. Physiol.* **66**, 431–36 (1965)
Cameron, I. L., G. M. Padilla, and O. L. Miller, Jr.	Macronuclear cytology of synchronized *Tetrahymena pyriformis*	*J. Protozool.* **13**, 336–41 (1966)
Caro, L. G.	Progress in high-resolution autoradiography	Pp. 173–90 in *Progress in Biophysics and Molecular Biology*, vol. 16 (ed. by J. A. V. Butler and H. E. Huxley), Pergamon, New York, 1966
Case, M. E., H. E. Brockman, and F. J. de Serres	Further information on the origin of the Yale and Oak Ridge wild-type strains of *N. crassa*	*Neurospora Newsletter* **8**, 25–26 (1966)
Congdon, C. C	Comment on cover	*Exptl. Hematol.* **10**, v–vi (1966)
Congdon, C. C	The destructive effect of radiation on lymphatic tissue	*Cancer Res.* **26**, 1211–20 (1966)
Congdon, C. C, Joan Wright Goodman, and J. W. Ferrebee	Effect of thymus transplantation on radiation chimeras	Pp. 411–12 in *The Year Book of Radiology*, 1965–1966 Series (ed. by J. F. Holt, W. M. Whitehouse, and H. B. Latourette), Year Book Medical Publishers, Chicago, 1966
Cosgrove, G. E.	Late somatic effects of radiation exposure in laboratory mice	*J. Am. Vet. Radiol. Soc.* **4**, 95–102 (1965)
Cosgrove, G. E.	The trematodes of laboratory primates	*Lab. Animal Care* **16**, 23–39 (1966)
Cudkowicz, Gustavo, and J. H. Stimpfling	Lack of expression of parental isoantigen(s) in F_1 hybrid mice	Pp. 144–48 in *Proceedings of the Tenth Congress of the International Society of Blood Transfusion, Stockholm, September 3–8, 1964–Part 1: Concepts of Autoimmunity and Transplantation Immunology* (ed. by L. Holländer), S. Karger, Basel/New York, 1965
de Serres, F. J.	The utilization of leaky *ad-3* mutants of *Neurospora crassa* in heterokaryon tests for allelic complementation	*Mutation Res.* **3**, 3–12 (1966)
Dunaway, P. B., and G. E. Cosgrove	A skin carcinoma in a cotton rat	*Am. Midland Naturalist* **75**, 535–37 (1966)
Epler, J. L.	Ethyl methanesulfonate–induced lethals in Drosophila frequency-dose relations and multiple mosaicism	*Genetics* **54**, 31–36 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Franklin, J. A., M. L. Simmons, and G. E. Cosgrove	A pathogen survey in the Kansas cottontail	*Bull. Wildlife Disease Assoc.* **2**, 52–53 (1966)
Füchtbauer, Walter, and Peter Mazur	Kinetics of the ultraviolet-induced dimerization of thymine in frozen solutions	*Photochem. Photobiol.* **5**, 323–35 (1966)
Fujimura, R. K.	Effect of colicine E2 on the biosynthesis of bacteriophage R17	*J. Mol. Biol.* **17**, 75–85 (1966)
Goodman, Joan Wright	Similarities and differences in the specific immunological unresponsiveness between genetically tolerant mice and those made tolerant at birth	Pp. 149–54 in *Proceedings of the Tenth Congress of the International Society of Blood Transfusion, Stockholm, September 3–8, 1964–Part 1: Concepts of Autoimmunity and Transplantation Immunology* (ed. by L. Holländer), S. Karger, Basel/New York, 1965
Goodman, Joan Wright, and Mysa C. Mathews	Erythropoiesis in chimeric mice of several donor-host combinations	*Am. J. Physiol.* **210**, 563–68 (1966)
Graebe, J. E., and G. D. Novelli	A practical method for large-scale plant tissue culture	*Exptl. Cell Res.* **41**, 509–20 (1966)
Graebe, J. E., and G. D. Novelli	Amino acid incorporation in a cell-free system from submerged tissue cultures of *Zea mays* L	*Exptl. Cell Res.* **41**, 521–34 (1966)
Gross, J. D., and L. G. Caro	DNA transfer in bacterial conjugation	*J. Mol. Biol.* **16**, 269–84 (1966)
Gude, W. D., D. A. Jones, and A. C. Upton	Inclusion of film-mounted histologic sections in IBM data-processing cards for simplified filing and reference	*Stain Technol.* **41**, 194–96 (1966)
Haber, A. H.	Heat inactivation of gibberellic acid-sensitivity of lettuce seed germination	*Plant Cell Physiol. (Tokyo)* **6**, 565–69 (1965)
Haber, A. H.	Meeting report on 21st Southern Section Mtg. of the Am. Soc. Plant Physiol., Dallas, Feb. 1–3, 1965	*Bioscience* **6**, 427–28 (1965)
Haber, A. H.	Meeting report on 21st Southern Section Mtg. of the Am. Soc. Plant Physiol., Dallas, Feb. 1–3, 1965	*Plant Physiol.* **40**, 588 (1965)
Hanna, M. G., Jr., C. C Congdon, and C. J. Wust	Effect of antigen dose on lymphatic tissue germinal center changes	*Proc. Soc. Exptl. Biol. Med.* **121**, 286–90 (1966)
Hanna, M. G., Jr., D. C. Swartzendruber, and C. C Congdon	Morphologic changes in spleen lymphatic tissue during antibody production	*Exptl. Mol. Pathol.* (suppl. 3), 75–87 (1966)
Harrison, A. P., Jr.	Thymine incorporation and metabolism by various classes of thymine-less bacteria	*J. Gen. Microbiol.* **41**, 321–33 (1965)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Heddle, John A., and J. E. Trosko	Is the transition from chromosome to chromatid aberrations the result of the formation of single-stranded DNA?	*Exptl. Cell Res.* **42**, 171–77 (1966)
Heddle, John A., and Sheldon Wolff	Estimation of the rejoining distance for chromosome exchanges induced in *Drosophila* sperm by combined doses of x rays and neutrons	*Intern. J. Radiation Biol.* **10**, 207–10 (1966)
Hollaender, Alexander	Symposium on Differentiation and Growth of Hemoglobin- and Immunoglobulin-Synthesizing Cells (and Introduction)	*J. Cell. Physiol.* (suppl. 1) **67**, 215 pp. (244 incl. index)
Hollaender, Alexander, S. F. Carson, and J. L. Liverman	Biology Division Semiannual Report for Period Ending January 31, 1966	ORNL-3922
Igarashi, Satomi	Temperature-sensitive mutation in *Paramecium aurelia*. I. Induction and inheritance	*Mutation Res.* **3**, 13–24 (1966)
Igarashi, Satomi	Temperature-sensitive mutation in *Paramecium aurelia*. II. Modification of the mutation frequency by pre- and postirradiation conditions	*Mutation Res.* **3**, 25–33 (1966)
Jenkins, V. K., T. T. Odell, Jr., and A. C. Upton	Effect of sex hormones on susceptibility to viral transmission of myeloid leukemia in the mouse	*Cancer Res.* **26**, 454–58 (1966)
Kimball, R. F.	Repair of premutational damage	Pp. 135–66 in *Advances in Radiation Biology*, vol. 2 (ed. by L. G. Augenstein, Ronald Mason, and Max Zelle), Academic, New York and London, 1966
Kondo, Sohei, and John Jagger	Action spectra for photoreactivation of mutation to prototrophy in strains of *Escherichia coli* possessing and lacking photoreactivating-enzyme activity	*Photochem. Photobiol.* **5**, 189–200 (1966)
Kretchmar, A. L., and C. C Congdon	The metabolic problem in secondary disease	Pp. 347–52 in *La Greffe des Cellules Hématopoïétiques Allogéniques, Colloques Internationaux du Centre National de la Recherche Scientifique*, No. 147, Centre National de la Recherche Scientifique, Paris, 1965
Magni, G. E., R. C. von Borstel, and C. M. Steinberg	Super-suppressors as addition-deletion mutations	*J. Mol. Biol.* **16**, 568–70 (1966)
Makinodan, Takashi	Changes in immunobiological processes caused by radiation	Pp. 303–33 in *Encyclopedia of Medical Radiology* (Radiation Biology, part 2, ed. by A. Zuppinger) (ed. by L. Diethelm *et al.*), Springer-Verlag, Berlin, Heidelberg, New York, 1966

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Mattingly, Ellen	Differences in mitotic cycle in various tissues of *Vicia faba* roots as revealed by 5-amino uracil synchronization of cell division	*Exptl. Cell Res.* **42**, 274–80 (1966)
Mazur, Peter	Theoretical and experimental effects of cooling and warming velocity on the survival of frozen and thawed cells	*Cryobiology* **2**, 181–92 (1966)
McGrath, R. A., R. W. Williams, and D. C. Swartzendruber	Breakdown of DNA in x-irradiated *Escherichia coli*	*Biophys. J.* **6**, 113–22 (1966)
Morita, Toshiteru, and E. H. Perkins	A simple quantitative method to assess the in vitro engulfing and degradative potentials of mouse peritoneal phagocytic cells	*RES, J. Reticuloendothelial Soc.* **2**, 406–19 (1965)
Muhammed, Amir	Studies on the yeast photoreactivating enzyme. I. A method for the large scale purification and some properties of the enzyme	*J. Biol. Chem.* **241**, 516–23 (1966)
Nelson, Bill, G. E. Cosgrove, and Nazareth Gengozian	Diseases of an imported primate, *Tamarinus nigricollis*	*Lab. Animal Care* **16**, 255–75 (1966)
Olivieri, Gregorio, and J. G. Brewen	Evidence for nonrandom rejoining of chromatid breaks and its relation to the origin of sister-chromatid exchanges	*Mutation Res.* **3**, 237–48 (1966)
Padilla, G. M., P. A. Van Dreal, and N. G. Anderson	Studies on synchronized cells: radiation-induced division delay in the flagellate *Astasia longa*	*Radiation Res.* **28**, 157–65 (1966)
Pavan, Crodowaldo	Nucleic acid metabolism in polytene chromosomes and the problem of differentiation	Pp. 222–41 in *Brookhaven Symposia in Biology, No. 18: Genetic Control of Differentiation*, Brookhaven National Laboratory, Upton, N. Y., 1965
Pavan, Crodowaldo, and Renato Basile	Chromosome changes induced by infections in tissues of *Rhynchosciara angelae*	*Science* **151**, 1556–58 (1966)
Pavan, Crodowaldo, and Renato Basile	Invertebrate pathology, cytology, and development	*J. Invertebrate Pathol.* **8**, 131–32 (1966)
Payne, J. A., and G. E. Cosgrove	Tissue changes following *Cuterebra* infestation in rodents	*Am. Midland Naturalist* **75**, 205–13 (1966)
Perkins, E. H., Paul Nettesheim, and Toshiteru Morita	Radioresistance of the engulfing and degradative capacities of peritoneal phagocytes to kiloroentgen x-ray doses	*RES, J. Reticuloendothelial Soc.* **3**, 71–82 (1966)
Pfuderer, Peter, and D. C. Swartzendruber	The configuration of isolated polysomes	*J. Cell Biol.* **30**, 193–97 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
Popp, R. A., Judith G. Heddle, R. E. Canning, and R. C. Allen	Some physical and chemical properties of albumin esterase and albumin from mouse serum	*Biochim. Biophys. Acta* **115**, 113–20 (1966)
Randolph, M. L., and J. L. Hosszu	Corrections for inherent timing errors in irradiations	*Health Phys.* **12**, 971–73 (1966)
Regan, J. D., and E. H. Y. Chu	A convenient method for assay of DNA synthesis in synchronized human cell cultures	*J. Cell Biol.* **28**, 139–43 (1966)
Ritossa, F. M., and Piero Cammarano	Isolation and properties of ribosomes from D. melanogaster	*Drosophila Info. Serv.* **41**, 129–30 (1966)
Russell, Liane B., and Clyde S. Montgomery	Radiation-sensitivity differences within cell-division cycles during mouse cleavage	*Intern. J. Radiation Biol.* **10**, 151–64 (1966)
Russell, Liane B., and Florence N. Woodiel	A spontaneous mouse chimera formed from separate fertilization of two meiotic products of oogenesis	*Cytogenetics* **5**, 106–19 (1966)
Setlow, R. B.	Cyclobutane-type pyrimidine dimers in polynucleotides	*Science* **153**, 379–86 (1966)
Setlow, R. B., and W. L. Carrier	Pyrimidine dimers in ultraviolet-irradiated DNA's	*J. Mol. Biol.* **17**, 237–54 (1966)
Stapleton, G. E., and E. B. Darden	The ORNL Space Biology Program, Annual Report, Period Ending June 30, 1965	ORNL-TM-1217
Swartzendruber, D. C.	The fine structure of lymphatic tissue germinal centers: reticular remnant after injury	*Am. J. Pathol.* **48**, 613–26 (1966)
Swenson, P. A., and R. B. Setlow	Effect of ultraviolet radiation on macromolecular synthesis in *Escherichia coli*	*J. Mol. Biol.* **15**, 201–19 (1966)
Thomas, Ruth E., and P. A. Roberts	Comparative frequency of x-ray induced crossover-suppressing aberrations recovered from oocytes and sperm of *Drosophila melanogaster*	*Genetics* **53**, 855–62 (1966)
Upton, A. C.	Cancer research 1964: thoughts on contributions of radiation biology	Pp. 391–92 in *The Year Book of Radiology*, 1965–1966 Series (ed. by J. F. Holt, W. M. Whitehouse, and H. B. Latourette), Year Book Medical Publishers, Chicago, 1966
Upton, A. C.	Pathologic effects	Pp. 126–62 in *The Biological Basis of Radiation Therapy* (ed. by E. E. Schwartz), Lippincott, Philadelphia and Toronto, 1966
Upton, A. C.	Radiobiological aspects of the supersonic transport	*Health Phys.* **12**, 209–26 (1966)
von Borstel, R. C.	Effects of radiation on cells	Pp. 60–125 in *The Biological Basis of Radiation Therapy* (ed. by E. E. Schwartz), Lippincott, Philadelphia and Toronto, 1966
von Borstel, R. C.	Yeast genetics	*Science* **152**, 1287–88 (1966)

AUTHOR(S)	TITLE OF ARTICLE	PUBLICATION
von Borstel, R. C., D. M. Prescott, and F. J. Bollum	Incorporation of nucleotides into nuclei of fixed cells by DNA polymerase	*J. Cell Biol.* **29**, 21–28 (1966)
Walburg, H. E., Jr., Edna I. Mynatt, and D. M. Robie	The effect of strain and diet on the thirty-day mortality of x-irradiated germfree mice	*Radiation Res.* **27**, 616–29 (1966)
Whitson, G. L., G. M. Padilla, and W. D. Fisher	Cyclic changes in polyribosomes of synchronized *Tetrahymena pyriformis*	*Exptl. Cell Res.* **42**, 438–46 (1966)
Yamada, Tuneo	Control of tissue specificity: the pattern of cellular synthetic activities in tissue transformation	*Am. Zool.* **6**, 21–31 (1966)
Zimmerman, B. K.	Purification and properties of deoxyribonucleic acid polymerase from *Micrococcus lysodeikticus*	*J. Biol. Chem.* **241**, 2035–41 (1966)

LECTURES

During the period February 1 to July 31, 1966, members of the Division presented 277 lectures and speeches. This figure compares with 247 for the corresponding period of 1965 and with 199 for the last reporting period, August 1, 1965, to January 31, 1966. Of the 277 listed below, 128 were given at professional society meetings (including several read by title); 127 were given at the invitation of universities, laboratories, scientific institutions, and professional groups or clubs in the United States and abroad; and 22 were 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 of that name (see Contents).

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Adler, H. I.	Genetic control of cell division and recovery from radiation damage	University of Frankfurt, Frankfurt, Germany
	Genetic control of radiation sensitivity in bacteria	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
	Repair of damage to the cell division mechanism in bacteria	Chester Beatty Research Institute, London
Adler, H. I. (W. D. Fisher, Alice A. Hardigree, and G. E. Stapleton)	Control of cell division in irradiated bacteria	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Albright, J. F.	Immunological competence of cells as revealed by competition of antigens	The Southwest Center for Advanced Studies, Dallas

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Albright, J. F. (and Takashi Makinodan)	Growth and senescence of antibody-forming cells	Symposium on Differentiation and Growth of Hemoglobin- and Immunoglobulin-Synthesizing Cells, Gatlinburg, Tenn. (ORNL Biology Division Research Conference)
Allen, R. C.	High resolution electrophoresis in polyacrylamide gel	1. Stanford University, Stanford, Calif. 2. Fort Detrick, Frederick, Md.
	Separation and characterization of erythropoietin by vertical flat bed discontinuous electrophoresis in polyacrylamide gel	University of Tennessee Medical College, Memphis
Allen, R. C. (J. W. Fisher and Dorothy J. Moore)	Purification of sheep and mouse erythropoietin by vertical flat bed discontinuous electrophoresis in acrylamide gel	Erythropoietin Conference, New York Academy of Sciences, New York
Allen, R. C. (R. T. Jones, G. W. Fortner, and J. H. Chandler, Jr.)	Quantitative and qualitative relationships of an intestinal microflora in specifically contaminated mice of germfree origin	Am. Soc. Microbiol., Round-Table, Los Angeles
Anderson, N. G.	Cell fractionation in zonal centrifuges	Lipid Meeting, ORINS Medical Division, Oak Ridge, Tenn.
	The MAN program: the development of new basic tools for cell fractionation	Assoc. State and Territorial Public Health Laboratory Directors, Atlanta, Ga.
	Physical approaches to cellular substructure: the MAN program	Scientific Research Society of America, Oak Ridge, Tenn.
	Research on the liquid centrifuge	U. S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
	Separation systems for the MAN program	Physics Division Seminar, ORNL, Oak Ridge, Tenn.
	The zonal centrifuge	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tenn.
Anderson, N. G. (J. G. Green and C. D. Scott)	Chromatography systems for the MAN program	Symposium on Modern Applications of Column Chromatography, University of Wisconsin, Madison
Anderson, N. G. (D. A. Waters, C. E. Nunley, and G. B. Cline)	Two new simple zonal centrifuge rotors: B-XIV and B-XV [*Federation Proc.* **25**, 421 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Arnold, W. A.	The light reaction of photosynthesis	University of Tennessee, Knoxville
Baccarini, Iracema M. (A. C. Upton and T. T. Odell, Jr.)	Histological, histochemical, and autoradiographic studies of the estrous cycle in the mouse	First Pan American Congress of Anatomy, Mexico City
Barber, A. A. (and Kenneth Allen)	Association of phosphorylase activity with liver glycogen particles separated by rate-zonal centrifugation [*Federation Proc.* **25**, 575 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Barnett, W. E.	The genetic code in *Neurospora*	Illinois State University, Normal
	Multiple aminoacyl-RNA synthetase	University of Georgia, Athens
	The roles of tRNA in protein synthesis (three lectures)	Nucleic Acids Course, University of Georgia, Athens
Barnett, W. E. (and J. L. Epler)	Multiple aminoacyl-RNA synthetase systems [*Federation Proc.* **25**, 520 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
	Multiple aminoacyl-RNA synthetase systems and the genetic code in *Neurospora*	Cold Spring Harbor Symp. Quant. Biol. XXXI: The Genetic Code, Cold Spring Harbor, N. Y.
Bender, M. A (J. B. Davidson and M. A. Kastenbaum)	Chromosome analysis	Symposium on Use of Computers in Analysis of Experimental Data and Control of Nuclear Facilities, Argonne National Laboratory, Argonne, Ill.
Bender, M. A (and P. Carolyn Gooch)	Human blood experiments in the Gemini series [*Radiation Res.* **27**, 489 (1966)]	Radiation Research Society (14th Annual Meeting), Coronado, Calif.
Bender, R. S. (and E. B. Darden, Jr.)	A facility for proton irradiation of mice at the Oak Ridge isochronous cyclotron [*Radiation Res.* **27**, 539 (1966)]	Radiation Research Society (14th Annual Meeting), Coronado, Calif.
Bennett, Michael (and Gustavo Cudkowicz)	Deficient production of nonerythroid cells by transplanted marrow of anemic WW^V mice [*Federation Proc.* **25**, 296 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Berg, Claire M.	Inverted and noninverted transpositions in the genome of *Escherichia coli* K-12 [*Bacteriol. Proc.*, pp. 26–27 (1966)]	Am. Soc. Microbiol. (66th Annual Meeting), Los Angeles
	Replication and genetic exchange in the genome of *Escherichia coli* K-12	1. Stanford University, Stanford, Calif. 2. University of California, Berkeley 3. University of Washington, Seattle 4. Rockefeller University, New York
Bosma, M. J. (Takashi Makinodan and H. E. Walburg, Jr.)	A comparative study of the immune potential of germfree and conventional mice [*Federation Proc.* **25**, 547 (1966)]	1. Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J. 2. The Institute for Cancer Research, Philadelphia
Brewen, J. G.	Kinetics of induced chromosome aberrations	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
Brewen, J. G. (Gregorio Olivieri and J. G. Brewen)	Evidence suggestive to the origin of sister chromatid exchanges	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Brewen, J. G. (and Gregorio Olivieri)	Radiation-induced chromatid aberrations and complications introduced by stage sensitivity and mitotic delay (read by title)	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Stage sensitivity and complications introduced by mitotic delay	Colloquium on Changes in the Cell Cycle Affecting Radiosensitivity, Casaccia, Rome

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Caro, L. G.	The attachment of f1 bacteriophage to male strains of *Escherichia coli* [*Bacteriol. Proc.*, pp. 111–12 (1966)]	Am. Soc. Microbiol. (66th Annual Meeting), Los Angeles
	F-pili and f-1 phage	Kansas State University, Manhattan
	Principles of high-resolution autoradiography	International Symposium on Electron Microscopy and Cytochemistry, Leiden, The Netherlands
Charamella, L. J. (and Roy Curtiss III)	The *pro-lac* region of the *Escherichia coli* K-12 genome [*Bacteriol. Proc.*, p. 27 (1966)]	Am. Soc. Microbiol. (66th Annual Meeting), Los Angeles
Chu, E. H. Y. (and J. E. Trosko)	Inhibition of DNA synthesis by ultraviolet irradiation in mammalian cells in vitro	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Clapp, N. K.	Chemical carcinogenesis with nitrosamines	U. S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
	Radiation and chemical carcinogenesis research	Rotary Club, Etowah, Tenn.
	Radiation and radiomimetic chemicals in biological research	Kiwanis Club, Athens, Tenn.
Clapp, N. K. (and A. W. Craig)	Early liver changes in mice treated with dialkylnitrosamines [*Cancer Res.* **7**, 14 (1966)]	Am. Assoc. Cancer Res., Denver, Colo.
Clapp, N. K. (and E. W. Kienholz)	Radioprotection in chick embryos from hens fed zinc [*Radiation Res.* **27**, 511 (1966)]	Radiation Research Society (14th Annual Meeting), Coronado, Calif.
Cohn, W. E.	Rapid chromatographic analysis of nucleotide material	1. Ames Research Center, Moffett Field, Calif. 2. University of Oslo, Oslo
Congdon, C. C	1. Experimental design studies on secondary disease in radiation chimeras 2. ORNL Biology Division research programs	Interlochen Arts Academy, Interlochen, Mich.
	Stem cells and lymphatic tissues	Conference on the Hemopoietic Stem Cell, Hinterzarten, Germany
Congdon, C. C (W. D. Gude, C. C Congdon, and G. E. Cosgrove)	Splenic fibrinoid necrosis in mouse radiation chimeras [*Federation Proc.* **25**, 232 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Congdon, C. C (and M. G. Hanna, Jr.)	Comparison of existing theories on the function of germinal centers [*Exptl. Hematol.* **11**, 3 (1966)]	Conference on Germinal Centers of Lymphatic Tissues, Bern, Switzerland
Congdon, C. C (M. A. Kastenbaum and D. A. Gardiner)	Factors that influence mortality from secondary disease in radiation chimeras	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Cook, Dorothy S.	Ribosomal RNA in growing and nongrowing epithelium	Developmental Biology Conference for New England, Storrs, Conn.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Corbett, J. R.	Separation of lysosomes by the B-XV zonal centrifuge rotor [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 90]	Biophysical Society (10th Annual Meeting), Boston
	Separation of rat liver cell components in the B-XV zonal centrifuge rotor [*Federation Proc.* **25**, 759 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
	The use of the zonal centrifuge to purify lysosomes	Vanderbilt University, Nashville, Tenn.
Cosgrove, G. E. (M. L. Davis and E. B. Darden, Jr.)	Late hematologic effects of 14 Mev neutrons in mice [*Radiation Res.* **27**, 530 (1966)]	Radiation Research Society (14th Annual Meeting), Coronado, Calif.
Cudkowicz, Gustavo	A new class of genes affecting histocompatibility of hematopoietic grafts in irradiated mice	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Cudkowicz, Gustavo (Michael Bennett and Gustavo Cudkowicz)	Functional and morphological characterization of stem cells: the unipotential role of "lymphocytes" of mouse marrow	International Symposium on The Lymphocyte in Immunology and Haemopoiesis, Bristol, England
Curtiss, Roy, III	Reanalysis of the steps during bacterial conjugation in *Escherichia coli*	1. School of Medicine, Western Reserve University, Cleveland, Ohio 2. Yale University, New Haven, Conn.
Curtiss, Roy, III (and L. G. Caro)	Role of donor pili in specific pair formation and chromosome transfer in *Escherichia coli* K-12 [*Bacteriol. Proc.*, p. 27 (1966)]	Am. Soc. Microbiol. (66th Annual Meeting), Los Angeles
Darden, E. B., Jr.	Effect of radiation on biological organisms	U. S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
de Serres, F. J.	The correlation between genetic alteration and complementation pattern among nitrous acid-induced mutants of *Neurospora crassa*	1. Institute of General Microbiology, University of Bern, Bern, Switzerland 2. University of Sheffield, Sheffield, England
de Serres, F. J. (H. V. Malling and B. B. Webber)	Dose-rate dependence of x-ray-induced nuclear inactivation in heterokaryotic conidia and forward-mutation in the *ad-3* region of *Neurospora crassa*	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Donnellan, J. E., Jr. (R. S. Stafford and R. B. Setlow)	Effects of uv irradiation on *Bacillus megaterium* [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 112]	Biophysical Society (10th Annual Meeting), Boston
Ehling, U. H.	Mutation research in mammals	The Free University, Berlin
	Mutations affecting the skeleton in offspring of irradiated male mice	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Einstein, J. R.	The combination of isomorphous replacement and anomalous scattering data in phase determination for protein crystals	1. Congress of the International Union of Crystallography, Moscow (read by title) 2. The Center for Crystallographic Research, Buffalo, N. Y. 3. Harvard University, Cambridge, Mass.
	A possible method for solving protein structures	American Crystallographic Association, Austin, Tex.
Epler, J. L.	Genetic code in *Neurospora*	University of Delaware, Newark
	Transfer of genetic information	Millersville State College, Millersville, Pa.
	Protein synthesis	National Science Foundation Summer Institute, Le Moyne College, Memphis, Tenn.
Evans, John (and Stanfield Rogers)	Studies of the nuclear histones of Shope virus induced rabbit papillomas [*Federation Proc.* **25**, 478 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Finamore, F. J.	Nucleic acid and nucleotide metabolism in normal and x-irradiated brine shrimp embryos	1. Wittenberg University, Springfield, Ohio 2. Sam Houston State College, Huntsville, Tex. 3. Vanderbilt University, Nashville, Tenn. 4. Tuskegee Institute, Tuskegee Institute, Ala. 5. Florida State University, Tallahassee 6. University of Georgia, Athens
	Nucleic acid metabolism in normal and x-irradiated brine shrimp embryos	University of Windsor, Windsor, Ontario, Canada
Fisher, W. D.	The applications of zonal centrifugation to biology	Duke University, Durham, N. C.
Foard, D. E.	The use of γ plantlets in studies of growth and development	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
	Radiation studies in plant physiology	U. S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Fujimura, R. K. (and Elliot Volkin)	Inhibitory action of colicine E2 on bacteriophage R17-infected cells [*Bacteriol. Proc.*, p. 113 (1966)]	Am. Soc. Microbiol. (66th Annual Meeting), Los Angeles
Gooch, P. Carolyn (M. A Bender and P. Carolyn Gooch)	Cytogenetic methods: somatic chromosome aberrations in occupationally irradiated humans	Symposium on Accidental Irradiation at Place of Work, Nice, France (sponsored by Euratom)

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Gooch, P. Carolyn (M. A Bender and Sohei Kondo)	Experiments on human somatic chromosome aberration production in connection with the Gemini manned space flight series (read by title)	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Gude, W. D.	Autoradiography	Medical Radioisotope Course, Medical Division, ORINS, Oak Ridge, Tenn.
	Current research in the Pathology and Physiology section of the Biology Division, ORNL	U. S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Haber, A. H.	Cellular interpretations of plant morphogenesis	Tennessee Technological University, Cookeville
	Radiation effects on germination	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
Haber, A. H. (and M. L. Randolph)	Effects of wetting and redrying on biological and radiological properties of lettuce seeds	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Radiation-induced and endogenous electron spin resonance signals in lettuce seeds [*Proc. Assoc. So. Agri. Workers, Inc., 63rd Annual Convention, 1966*, p. 288]	Am. Soc. Plant Physiol. (Southern Section), Jackson, Miss.
Hanna, M. G., Jr. (Takashi Makinodan and W. D. Fisher)	Lymphatic tissue localization of I^{125} labeled heterologous and isologous macroglobulins [*Exptl. Hematol.* **11**, 8 (1966)]	Conference on Germinal Centers of Lymphatic Tissues, Bern, Switzerland
Hanna, M. G., Jr. (D. C. Swartzendruber and C. C Congdon)	Morphologic and autoradiographic studies of spleen white pulp germinal centers during an immune reaction [*Exptl. Hematol.* **11**, 16–17 (1966)]	Conference on Germinal Centers of Lymphatic Tissues, Bern, Switzerland
Harris, W. W. (E. L. Candler, Jr., N. G. Anderson, and C. T. Rankin, Jr.)	Zonal centrifugation of tumor tissue [*Cancer Res.* **7**, 29 (1966)]	Am. Assoc. Cancer Res., Denver, Colo.
Harrison, A. P., Jr.	Differences in thymine metabolism and incorporation by various thymineless bacteria [Abstr. of papers, 10th Mtg., Biophys. Soc., 1966, p. 62]	Biophysical Society (10th Annual Meeting), Boston
Hellman, Alfred (J. G. Farrelly and D. H. Martin)	Some virucidal and cytocidal properties of dimethyl sulfoxide [*Bacteriol. Proc.*, 114–15 (1966)]	Am. Soc. Microbiol. (66th Annual Meeting), Los Angeles
Hellman, Alfred (J. D. Regan and J. G. Farrelly)	Aminoacyl-sRNA synthetase systems of a viral infected cell	Tissue Culture Association, San Francisco

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Hollaender, Alexander	1. Opening Remarks 2. Closing Remarks	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Closing Remarks	Colloquium on Changes in the Cell Cycle Affecting Radiosensitivity, Casaccia, Rome
	Developments in biological research	University of Texas, Austin
Holten, D. D. (and F. T. Kenney)	Mechanisms controlling hepatic enzyme synthesis [*Federation Proc.* **25**, 285 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Jacobson, K. B.	Effect of t-RNA on hemoglobin synthesis	Cold Spring Harbor Symp. Quant. Biol. XXXI: The Genetic Code. Cold Spring Harbor, N. Y.
	Isozymes of alcohol dehydrogenase of *Drosophila*	1. University of North Carolina, Chapel Hill 2. University of Georgia, Athens 3. Duke University, Durham, N. C.
Jacobson, K. B. (E. H. Grell and J. B. Murphy)	Alcohol dehydrogenase of *Drosophila melanogaster* [*Federation Proc.* **25**, 713 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Jones, R. T. (Roy Curtiss III, R. T. Jones, R. C. Allen, and Paula E. Harris)	Genetically marked *Escherichia coli* strains for studies on intestinal flora in mice [*Bacteriol. Proc.*, 38 (1966)]	Am. Soc. Microbiol. (66th Annual Meeting), Los Angeles
Jurand, Artur (Tuneo Yamada and D. H. Reese)	Sequential changes in the pattern of ribosomes during Wolffian lens regeneration	Lens Differentiation Conference, Morgantown, W. Va.
Kamrin, Michael	Recent physical studies of photosynthesis	University of Tennessee, Knoxville
Kelmers, A. D. (M. P. Stulberg and G. D. Novelli)	Preparation of highly purified phenylalanine and leucine transfer ribonucleic acids [*Federation Proc.* **25**, 520 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Kenney, F. T.	Hormonal control of liver enzyme synthesis	Washington University, St. Louis
	Hormonal regulation of hepatic enzyme synthesis	University of Maryland Medical School, Baltimore
Kimball, R. F.	A comparison of mutation induction by x rays and by triethylene melamine in *Paramecium*	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Variation of sensitivity to mutation induction during the cell cycle in *Paramecium*	Colloquium on Changes in the Cell Cycle Affecting Radiosensitivity, Casaccia, Rome
Kretchmar, A. L. (E. Rupp and C. C Congdon)	Response of radiation chimeras to repeated bleeding: reduced erythropoietic response during secondary syndrome	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Leibo, S. P. (and Peter Mazur)	Density gradient analysis of osmotic phenomena in bacteriophage T4B [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 65]	Biophysical Society (10th Annual Meeting), Boston
Levedahl, B. H. (and A. A. Barber)	Isolation of paramylon from *Euglena gracilis* var. *bacillarus* SML-1 by combined rate and isopycnic zonal centrifugation [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 42]	Biophysical Society (10th Annual Meeting), Boston
Lindsley, D. L. (S. A. Toledo F° and Chloe Camba Musatti)	The chromosomal basis of the sterility of sons of irradiated males in *Drosophila melanogaster*	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Lion, M. B.	Search for a mechanism to explain the high uv-sensitivity of 5-bromouracil substituted DNA	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Luippold, H. E.	Cytogenetic effects of radiations on *Vicia faba*	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
Makinodan, Takashi	Evidence for the commitment of cells to specific antibody synthesis	Gordon Research Conference on Cell Structure and Metabolism, Meriden, N.H.
	Growth and aging of the immune capacity of mice	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tenn.
Mattingly, Ellen	Diminution of aberration yield by treatment with actinomycin D (read by title)	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	1. Educational activities at the Biology Division	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
	2. Use of radioisotopes in cytology	
	The relationship of the nucleus and cytoplasm in problems of differentiation	1. Wright State Campus of Miami University and Ohio State University, Dayton 2. Vanderbilt University, Nashville, Tenn. 3. Texas A & M, College Station 4. Oklahoma State University, Stillwater 5. Washburn University of Topeka, Topeka, Kan.
Mazur, Peter (Walter Füchtbauer and Peter Mazur)	Kinetics of the ultraviolet-induced dimerization of thymine in frozen solutions [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 115]	Biophysical Society (10th Annual Meeting), Boston
McGrath, R. A.	Cellular repair mechanisms	1. The Free University, Berlin 2. Centro de Studi Nucleari della Casaccia, Comitato Nazionale per l'Energia Nucleare, Rome
	Correction on radiation damage	University of Grenoble, Grenoble, France

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
McGrath, R. A.	Repair of x-ray damage in *E. coli*	Medical Biological Laboratory RVO-TNO, Rijswijk, The Netherlands
	X-ray damage and repair in bacteria	Yale University School of Medicine, New Haven, Conn.
McGrath, R. A. (and R. W. Williams)	Evidence for repair in x-irradiated DNA [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 73]	Biophysical Society (10th Annual Meeting), Boston
	Repair of x-irradiated DNA	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
McLeish, John	Changes in nucleic acid and protein content during cellular development in the growing region of the root	University of Tennessee, Knoxville
	Nucleic acids in roots	Medical School of the University of Virginia, Charlottesville
	Quantitative relationships between DNA and RNA in isolated plant nuclei and nucleoli	Brookhaven National Laboratory, Upton, N. Y.
Morita, Toshiteru (and R. A. Brown)	Functional ribosomes in antibody-producing cells [*Federation Proc.* **25**, 369 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Morris, D. M.	Response of isologous and homologous hosts to a transplantable leukemia [*Anat. Record* **154**, 390 (1966)]	Am. Assoc. Anatomists, San Francisco
Muhammed, Amir	Purification of the photoreactivating enzyme	The Southwest Center for Advanced Studies, Dallas
Muhammed, Amir (and Jane K. Setlow)	Multiplicity reactivation and stimulation of recombination in T4 complexes irradiated at various uv wavelengths [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 111]	Biophysical Society (10th Annual Meeting), Boston
Nettesheim, Paul (and Carol J. Chadwick)	Improved diffusion chamber technique for studies of antibody response [*Federation Proc.* **25**, 369 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Nettesheim, Paul (and Takashi Makinodan)	Difference in the regenerating potential of immunologically competent spleen cells of unprimed and primed mice (read by title)	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Novelli, G. D.	Mechanisms in the biosynthesis of proteins (series of 3 lectures)	Biochemistry Course, University of Georgia, Athens
	Protein synthesis by regenerating liver	The Distinguished Lectureship Series, The Medical College of Georgia, Augusta
	Studies on tRNA	1. University of California, San Diego 2. Ames Research Center, Moffett Field, Calif.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Oakberg, E. F.	Effect of irradiation at 10 days of age on subsequent oocyte numbers and fertility of female mice	Colloquium on Radiation and Aging, Vienna
	Radiation response of spermatogonia in man	1. Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy 2. Norsk Hydro's Institute for Cancer Research, Montebello, Norway 3. Karolinska Institute, Stockholm
Odell, T. T., Jr.	Maturation of rat megakaryocytes	UT-AEC Agricultural Research Laboratory, Oak Ridge, Tenn.
Olivieri, Gregorio (and J. G. Brewen)	Evidence suggestive to the origin of sister chromatid exchanges	Colloquium on Changes in the Cell Cycle Affecting Radiosensitivity, Casaccia, Rome
Papaconstantinou, John	Molecular aspects of lens cell differentiation	1. Wilmer Institute, Johns Hopkins School of Medicine, Baltimore 2. Embryology Course at Woods Hole Marine Biological Station, Woods Hole, Mass.
	Ontogenetic approaches to immunity	Developmental Immunology Workshop No. 2, Gainesville, Fla.
Papaconstantinou, John (Emilia Julku and Dorothy Schenck)	The isolation and characterization of calf lens messenger RNA	Lens Differentiation Conference, Morgantown, W. Va.
Pavan, Crodowaldo	Changes in chromosomes induced by infections in *Rhynchosciara*	1. University of Texas, Austin 2. University of North Carolina, Chapel Hill
	The changes in polytene chromosomes induced by infections	University of Delaware, Newark
	Chromosomal changes induced by infections	University of Indiana, Bloomington
	Gene activity in different tissues of *Rhynchosciara*	Research Triangle Biology Club, Chapel Hill, N. C.
Pfuderer, Peter	Configuration studies on *E. coli* polysomes [*Federation Proc.* **25**, 781 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Popp, R. A.	Effects of prior treatment of recipients with x rays and allogeneic marrow on the duration of lag time between infusion and log phase proliferation of marrow cells in irradiated mice	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Hemoglobin variants in mice	1. Max-Planck Institute for Biochemistry, Munich, Germany 2. Institute of Genetics, Turin, Italy

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Popp, R. A.	Marrow transplantation in mice	1. Czechoslovak Academy of Sciences, Prague 2. Institute of Genetics, Turin, Italy
Rahn, R. O.	Thymine dimer formation in DNA at 77°K [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 83]	Biophysical Society (10th Annual Meeting), Boston
Randolph, M. L.	Formation, detection, and consequences of radiation-induced free radicals in biological material	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
Randolph, M. L. (and A. H. Haber)	Characterizations of native and induced ESR signals in seeds [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 84]	Biophysical Society (10th Annual Meeting), Boston
Randolph, M. L. (and M. G. Miller)	Endothermic decay of long-lived free radicals induced in amino acids by x rays	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Reese, D. H. (and Tuneo Yamada)	In vitro studies on Wolffian lens regeneration	Lens Differentiation Conference, Morgantown, W. Va.
Regan, J. D.	Alkaline phosphatases in human cell cultures	1. Florida State University, Tallahassee 2. Florida Atlantic University, Boca Raton 3. University of Miami, Miami, Fla.
Rogers, Stanfield	The Shope papilloma virus: a passenger in man and its significance [*Cancer Res.* **7**, 60 (1966)]	1. Am. Assoc. Cancer Res., Denver, Colo. 2. Symposium on Malignant Transformations, University of Chicago, Chicago
Russell, Liane B.	The mammalian X chromosome	1. Longwood College, Farmville, Va. 2. North Carolina State University, Raleigh
	Radiation sensitivity and developmental stage	Brookhaven National Laboratory, Upton, N. Y.
Russell, W. L.	Genetic effects of radiation	Conference on the Pediatric Significance of Peacetime Radioactive Fallout, San Diego, Calif.
	Recent results in mammalian radiation genetics	Brookhaven National Laboratory, Upton, N. Y.
Sassen, A. E.	Modifications of serum proteins in x-irradiated mice	University of Rochester, Rochester, N. Y.
Sassen, A. E. (Paul Nettesheim and D. J. Kroe)	Studies on differences in radiosensitivity of thymus and spleen [*Radiation Res.* **27**, 506 (1966)]	Radiation Research Society (14th Annual Meeting), Coronado, Calif.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Serrano, L. J.	Controlling variables for a radiobiology experiment on effects of low level irradiation	U. S. Army Nuclear Science Seminar, Oak Ridge, Tenn.
Setlow, Jane K.	The molecular basis of ultraviolet damage to DNA and its repair	University of Massachusetts, Amherst
	Reactivation of lethal uv damage in vivo and in vitro	Medical Biological Laboratory RVO-TNO, Rijswijk, The Netherlands
Setlow, Jane K. (and R. B. Setlow)	Reactivation of uv-inactivated DNA by further uv-irradiation in the presence of proflavine	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Reactivation of uv-inactivated transforming DNA by uv irradiation in the presence of proflavin [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. **72**]	Biophysical Society (10th Annual Meeting), Boston
Setlow, R. B.	1. The effects of ultraviolet radiation on DNA	University of Minnesota, St. Paul
	2. Mechanisms for correcting errors in DNA	
	Mechanisms for repair of DNA	Western Reserve University, Cleveland
	Photochemistry and photobiology of polynucleotides	University of Notre Dame, Notre Dame, Ind.
	The recovery of molecular damage to DNA	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Setlow, R. B.	Repair of DNA	Conference on Regulatory Mechanisms in Nucleic Acid and Protein Biosynthesis, Lunteren, The Netherlands
	Repair of uv damage in *E. coli*	Medical Biological Laboratory RVO-TNO, Rijswijk, The Netherlands
Setlow, R. B. (and W. L. Carrier)	The v-gene controls dimer excision in T4 phage [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 68]	Biophysical Society (10th Annual Meeting), Boston
Setlow, R. B. (W. L. Carrier and R. W. Williams)	Three levels of DNA repair in *E. coli* [*Science* **152**, 676 (1966)]	103rd Annual Meeting of the National Academy of Sciences, Washington, D. C.
Shugart, L. R. (and M. P. Stulberg)	Isolation of undermethylated $tRNA^{Phe}$ from *E. coli* [*Federation Proc.* **25**, 403 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Simmons, M. L.	Disease control in laboratory rodents after lethal and sub-lethal doses of ionizing x-irradiation	American Veterinary Medical Association Conference, Louisville, Ky.
Simmons, M. L. (and John Franklin)	Acute radiation mortality in mice inoculated with pigmented or nonpigmented *Pseudomonas* (read by title)	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Smith, L. H.	An in vitro bone marrow system for studying recovery from radiation injury	Conference on the Recovery of Irradiated Cells by Nucleic Acids, Herceg-Novi, Yugoslavia
Smith, L. H. (and T. W. McKinley, Jr.)	Erythropoietic capacity of x-irradiated spleen cells [*Radiation Res.* **27**, 504 (1966)]	Radiation Research Society (14th Annual Meeting), Coronado, Calif.
	Some in vitro factors which affect x-irradiated bone marrow cells	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Stapleton, G. E.	Types of radiation in biological research	ORINS-UT-AEC Radiobotany Institute, Oak Ridge, Tenn.
Stapleton, G. E. (and W. D. Fisher)	Ribosome pools and macromolecular synthesis in radiation resistant and sensitive *Escherichia coli*	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Steinberg, C. M.	P^{32} suicide	Vanderbilt University, Nashville, Tenn.
Stulberg, M. P.	Macromolecular interactions related to the first step of protein biosynthesis	University of Illinois School of Medicine, Chicago
	Some aspects of the preparation and interaction of phenylalanyl-RNA synthetase and tRNA	1. University of Minnesota, St. Paul 2. University of Minnesota, Minneapolis
Swartzendruber, D. C.	The fine structure of lymphatic tissue germinal centers after injury (read by title)	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Swenson, P. A. (and Barbara Hamkalo)	β-galactosidase formation in ultraviolet-irradiated sensitive and resistant strains of *Escherichia coli* B [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 72]	Biophysical Society (10th Annual Meeting), Boston
Trosko, J. E. (Margaret Kasschau, Lana Covington, and E. H. Y. Chu)	UV-induction of pyrimidine dimers during different phases of the cell cycle of mammalian cells [*Radiation Res.* **27**, 535 (1966)]	Radiation Research Society (14th Annual Meeting), Coronado, Calif.
Tyndall, R. L.	Viruses — a continuing challenge	1. Texas Woman's University, Denton 2. Jarvis Christian University, Hawkins, Tex. 3. Bradley University, Peoria, Ill. 4. Wright State Campus of Miami University and Ohio State University, Dayton, Ohio 5. College of Notre Dame of Maryland, Baltimore
Tyndall, R. L. (Ernestine Teeter, J. A. Otten, N. D. Bowles, and A. C. Upton)	Transformation in vitro and solid tumor formation associated with a murine leukemia virus	International Congress for Microbiology (9th Quadrennial), Moscow

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Upton, A. C.	Comparative observations on radiation carcinogenesis in man and animals	Fundamental Cancer Research Symposium (20th Annual), Houston
	Comparative observations on radiation-induced leukemias in man and mouse	Battelle Memorial Institute, Pacific Northwest Laboratories, Richland, Wash.
	Radiation leukemia	University of California Medical Center, San Francisco
	The relationship of experimental studies on cancer to the clinical problem of cancer	M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston
Upton, A. C. (J. W. Conklin and W. D. Gude)	Polyarteritis in aging RF mice [*Federation Proc.* **25**, 665 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Upton, A. C. (J. W. Conklin and R. A. Popp)	Age differences in susceptibility of mice to radiation-induced carcinogenesis and life-shortening	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Influence of age at irradiation on susceptibility to radiation-induced life-shortening in RF mice	Colloquium on Radiation and Aging, Vienna
Uziel, Mayo (and W. E. Cohn)	Chemical characterization of transfer RNA [*Federation Proc.* **25**, 784 (1966)] (read by title)	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
von Borstel, R. C.	Action of DNA and RNA polymerases on fixed cell preparations	Hebrew University, Jerusalem, Israel
	Chromosome breakage and dominant lethality	Israel Institute for Biological Research, Ness-Ziona, Israel
	Irradiation-of-male method of insect control	Meiners Entomological Society, St. Louis
	Super-suppressors	1. Yale University, New Haven, Conn. 2. Washington University, St. Louis 3. Weizmann Institute, Rehovoth, Israel 4. Hebrew University, Jerusalem, Israel
	The theory of radiation-induced dominant lethality in sperm	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
Walburg, H. E., Jr.	The Gnotobiotics Laboratory at Oak Ridge National Laboratory: effects of the microbial flora on aging in mice	University of Tennessee, Knoxville
Walburg, H. E., Jr. (and G. E. Cosgrove)	Aging in irradiated and unirradiated germ-free mice	Third International Congress of Radiation Research, Cortina d'Ampezzo, Italy
	Longevity as influenced by irradiation in germfree mice	Colloquium on Radiation and Aging, Vienna
Wallace, R. A.	Vitellogenesis	Embryology Training Course, Woods Hole, Mass.

SPEAKER [AND COAUTHOR(S)]	TITLE	PLACE PRESENTED
Wallace, R. A.	Vitellogenesis in vertebrates	University of Pennsylvania Medical School, Philadelphia
Whiting, P. W. (and Doris J. Bush)	Mass production of diapause larvae in *Mormoniella* [*ASB Bull.* **13**, 48 (1966)]	Assoc. SE Biol., Raleigh, N. C.
Wust, C. J.	Antibody synthesis (two lectures)	Auburn University, Auburn, Ala.
	Biochemical studies on antibody biosynthesis (three lectures)	Stetson University, Deland, Fla.
	Studies on polysomes in rat spleen during the immune response [*Federation Proc.* **25**, 369 (1966)]	Fed. Am. Soc. Exptl. Biol., Atlantic City, N. J.
Yamada, Tuneo	Cellular and subcellular events in Wolffian lens regeneration	Max-Planck Institut für Meeresbiologie, Wilhelmshaven, Germany
Yamada, Tuneo (Artur Jurand, Tuneo Yamada, and D. H. Reese)	Sequential changes in the pattern of ribosomes during Wolffian lens regeneration	Lens Differentiation Conference, Morgantown, W. Va.
Yamada, Tuneo (D. H. Reese and Tuneo Yamada)	In vitro studies on Wolffian lens regeneration	Lens Differentiation Conference, Morgantown, W. Va.
Zapisek, W. F. (and John Papaconstantinou)	Ribosomal breakdown in lens cell differentiation	Lens Differentiation Conference, Morgantown, W. Va.
Zimmerman, B. K.	The structure of copolymers of deoxyadenylate and deoxythymidylate synthesized by *M. lysodeikticus* DNA polymerase	Stanford University, Stanford, Calif.
	Studies on the DNA polymerase from *Micrococcus lysodeikticus*	Lawrence Radiation Laboratory, Livermore, Calif.
Zimmerman, B. K. (and G. R. Cassani)	The structure of copolymers of deoxyadenylate and deoxythymidylate synthesized by *M. lysodeikticus* DNA polymerase [Abstr. of papers, 10th Meeting, Biophys. Soc., 1966, p. 18]	Biophysical Society (10th Annual Meeting), Boston

VISITING LECTURERS

During the period February 1, 1966 – July 31, 1966, 52 lectures were given on the Biology Division Seminar Program by guest speakers from scientific institutions and universities in this country and abroad. Visiting lecturers from foreign countries included scientists from Argentina, Brazil, Czechoslovakia, England, Germany, and Israel.

SPEAKER	AFFILIATION	SUBJECT
John R. Baylis, Jr.	Department of Biological Sciences, Florida State University, Tallahassee	Mutation segregation studies in *Neurospora* and their implications
Werner Bode	Department of Physics, Kansas State University, Manhattan	Lysis inhibition
George B. Brown	Sloan-Kettering Institute, Rye, N.Y.	Oncogenic purines
Harris Busch	Department of Pharmacology, Baylor University, Houston	45S RNA and the nucleolus
A. B. Cairnie	Radiobiology Laboratory, University of California Medical Center, San Francisco	Cell proliferation in the small intestine of the rat
Donald R. Chadwick	Department of Health, Education, and Welfare, Washington, D.C.	The results of the nationwide x-ray exposure study
John Clark	Department of Biology, Massachusetts Institute of Technology, Cambridge	Solvation of macromolecules
John Costlow	Marine Biological Laboratory, Duke University, Beaufort, N.C.	The development of endocrine mechanisms in larval crab
J. E. Cummins	McArdle Laboratory for Cancer Research, University of Wisconsin, Madison	The role of newly synthesized protein in mitosis and in nuclear DNA replication and transcription during the cell cycle of *Physarum*
James W. Drysdale	Massachusetts Institute of Technology, Cambridge	Control mechanisms in the synthesis and turnover of liver ferritin
James D. Ebert	Department of Embryology, Carnegie Institution of Washington, Baltimore	Biochemistry and physiology of the developing heart
		Cellular differentiation and viral susceptibility
H. Feldman	University of Köln, Köln, Germany	Structure and function of transfer RNA's
J. R. S. Fincham	John Innes Institute, Bayfordbury, Hertfordshire, England	Genetic instability in antirrhinum
George Gassner	Department of Zoology, University of Texas, Austin	Ultrastructure of primary spermatocytes during prophase I in the scorpionfly, *Panorpa nuptialis* (Mecoptera)
N. Gengozian	Oak Ridge Associated Universities, Oak Ridge, Tenn.	Immunologic studies on the marmoset
C. W. Gilbert	Christie Hospital and Holt Radium Institute, Manchester, England	Some applications of computer calculations to chromosome and cell research
Ray Gilden	Flow Laboratories, Bethesda, Md.	Adenovirus tumor antigens
Edward Glassman	University of North Carolina, Chapel Hill	RNA metabolism in the brain during learning
Albert Grossman	New York University School of Medicine, New York	Regulation of tyrosine amino-transferase induction and subsequent inactivation
Richard C. Hard	Department of Pathology, University of Chicago, Chicago	Chimeric mice with donor liver cells

SPEAKER	AFFILIATION	SUBJECT
Fred Hartman	University of Illinois, Urbana	Evidence on the structure of ribonuclease obtained through the use of bifunctional protein reagents
E. A. D. Holmberg	Instituto de Investigaciones Hematologicas, Academia Nacional de Medicina de Buenos Aires, Buenos Aires	P^{32}-induced leukemia and "human" lymphomas transplanted into the mouse
		P^{32}-induced sterility in the mouse
F. Judge	Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, D. C.	Proposed federal legislation restricting use of animals in biomedical research
I. Kawakami	Department of Zoology, University of Michigan, Ann Arbor	Heterologous proteins as evocators in primary induction in amphibians
Edouard Kellenberger	Department of Physics, Kansas State University, Manhattan	Further studies on the morphopoiesis of the head of phage T4
L. G. Lajtha	Christie Hospital and Holt Radium Institute, Paterson Laboratories, Manchester, England	Clone size distribution in the study of growth in vitro
R. D. Lange	The University of Tennessee, Memorial Research Center, Knoxville	Use of silicone rubber membrane enclosures and erythropoietin antisera in studies of erythropoiesis
Francisco J. S. Lara	University of Sao Paulo, Sao Paulo, Brazil	Characterization of nucleic acids from *Rhynchosciara* salivary glands
Alena Lengerova	Institute of Experimental Biology and Genetics, Czechoslovak Academy of Sciences, Prague	Thymus and immunological self-recognition
Elizabeth Leuchars	Chester Beatty Research Institute, London	Studies on cells of thymic origin in radiation chimeras
Mario Luzzati	Department of Molecular Biophysics, Yale University, New Haven, Conn.	Specific inhibition of genetic recombination in *Saccharomyces cerevisiae*
M. Masters and W. Donachie	Medical Research Council, Hammersmith Hospital	Enzyme synthesis in synchronized bacteria
Lee Moore	Department of Physiology, Duke University Medical Center, Durham, N. C.	Anion permeability in frog skeletal muscle
Gisela Mosig	Department of Molecular Biology, Vanderbilt University, Nashville, Tenn.	Comparison of map distances with physics distances along the DNA molecule of phage T4
Michio Oishi	Princeton University, Department of Botany, Princeton, N. J.	Location of genetic loci for ribosomal transfer RNA on the *B. subtilis* chromosome
Crellin Pauling	Hansen Laboratories of Physics, Stanford University, Stanford, Calif.	DNA repair and thymineless death
Josip Pluščec	University of Chicago, Chicago	Biosynthesis of porphyrins

SPEAKER	AFFILIATION	SUBJECT
John K. Raison	U.S. Department of Agriculture, New Orleans	Protein synthesis and the possible utilization of phytate as a source of ATP in developing wheat endosperm
June Rothman	The Rockefeller Institute, New York	Diploidy in single-stranded DNA phage F1
John Scaife	Department of Bacteriology and Immunology, Harvard Medical School, Boston	F-prime formation in *E. coli* K12
Leonard T. Skeggs	Veterans Administration Hospital, Cleveland	Multiple analysis
David T. Suzuki	Department of Zoology, University of British Columbia, Vancouver, Canada	Negative interference in *Drosophila*
Milton W. Taylor	Department of Molecular and Cell Biology, University of California, Irving	Genetic localization of tryptophan transducing phage
C. A. Thomas	Department of Biophysics, Johns Hopkins University, Baltimore	The basic forms of the viral DNA molecule
D. Brynmor Thomas	University of Birmingham, Birmingham, England (currently Biology Div.)	Fetal hematopoiesis
		The transplantation potential of hepatic hemocytoblasts
Jacob Wahrman	Hebrew University of Jerusalem, Jerusalem, Israel	Whole-arm chromosome rearrangements in populations
Arnold Wishnia	Dartmouth Medical School, Hanover, N.H.	Hydrophobic interactions in proteins
Philip Woods	University of Delaware, Newark	Autoradiography of transfer RNA
Gerhard Zbinden	Hoffmann-La Roche, Inc., Nutley, N.J.	Drug toxicity, dilemma and challenge

SPEAKERS AT PROFESSIONAL MEETINGS, FALL–WINTER 1966

International Conference on Cryobiology, Sapporo, Japan – Peter Mazur

Seventeenth Annual Meeting of the American Institute of Biological Sciences, College Park, Md. – J. R. Corbett, G. E. Cosgrove, D. E. Foard (coauthor, A. H. Haber), and A. H. Haber

Electron Microscopy Society of America, San Francisco – W. W. Harris (coauthors, Frances L. Ball, Helen Coates, G. B. Cline, N. G. Anderson, and R. M. Chanock)

XX International Congress of Ophthalmology, Munich, Germany – John Papaconstantinou

XI International Congress of Hematology, Sydney – T. T. Odell, Jr. (coauthor, C. W. Jackson)

Eleventh Biennial Congress of the International Society of Blood Transfusion, Sydney – Peter Mazur and Joan Wright Goodman (coauthor, H. B. Wheeler)

International Symposium on Atherosclerosis and the Reticuloendothelial System, Como, Italy – E. H. Perkins (coauthors, Paul Nettesheim, Toshiteru Morita, and H. E. Walburg, Jr.)

Genetics Society of America, Chicago – D. E. Axelrod (coauthor, H. I. Adler), Roy Curtiss III (coauthor, L. J. Charamella), U. H. Ehling, Crodowaldo Pavan (coauthor, A. L. P. Perondini), P. A. Roberts (coauthors, R. F. Kimball and Crodowaldo Pavan), Liane B. Russell (coauthor, Clyde S. Montgomery), L. C. G. Simoes (coauthor, Crodowaldo Pavan), R. H. Smith (coauthor, Anna R. Whiting), and Gladys S. Van Pelt

Second International Biophysics Congress, Vienna – N. G. Anderson, L. G. Caro, Dorothy S. Cook, J. S. Cook, J. E. Donnellan (coauthor, R. S. Stafford), J. W. Longworth (coauthor, R. O. Rahn), John Papaconstantinou (coauthors, Emilia Julku and Dorothy M. Schenck), R. O. Rahn (coauthor, J. W. Longworth), and B. K. Zimmerman

Conference on Morphological and Biochemical Aspects of Cytodifferentiation, Zurich, Switzerland – Tuneo Yamada

152nd National Meeting of the American Chemical Society, New York – J. X. Khym

Seventeenth Annual Meeting of the Animal Care Panel, Chicago – J. O. Brick (coauthors, L. J. Serrano and N. L. Ensor), E. B. Darden, Jr. (coauthors, L. J. Serrano and L. W. Harris, Jr.), D. M. Robie (coauthors, M. L. Simmons and L. J. Serrano), and L. J. Serrano (coauthor, Carlene Amsbury)

Symposium on Gastrointestinal Radiation Injury, Richland, Wash. – G. E. Cosgrove (coauthors, H. E. Walburg, Jr., and A. C. Upton)

Second International Symposium on Cellular Chemistry, Otsu, Japan – F. J. Finamore

Sixth Annual Meeting of the American Society for Cell Biology, Houston – A. P. Harrison, Jr., R. F. Kimball, Ellen Mattingly, A. W. Prestayko (coauthor, W. D. Fisher), and G. L. Whitson (coauthor, J. G. Green)

Symposium on Enzymatic Aspects of Metabolic Regulation, Mexico City, Mex. – F. T. Kenney

1966 Autumn Meeting of the National Academy of Sciences, Durham, N.C. – H. I. Adler (coauthors, W. D. Fisher and G. E. Stapleton), W. E. Barnett (coauthor, D. H. Brown), R. F. Kimball, Takashi Makinodan (coauthors, Paul Nettesheim, Toshiteru Morita, and Carol J. Chadwick), W. L. Russell (coauthor, Elizabeth M. Kelly), C. M. Steinberg (coauthor, R. C. von Borstel), and R. C. von Borstel (coauthors, P. D. Panum and C. M. Steinberg)

Foreign Travel

Members of the Biology Division visited laboratories and other scientific institutions, presented papers at professional society meetings, and gave invited seminars in 20 foreign countries during the February–July 1966 reporting period. Countries visited were:

Europe – Austria, Belgium, Czechoslovakia, Denmark, England, France, Germany, Greece, Italy, The Netherlands, Norway, Russia, Scotland, Sweden, Switzerland, Yugoslavia

Asia – Israel

The Americas – Brazil, Canada, Mexico

Twenty-two Division members attended the Third International Congress of Radiation Research held at Cortina d'Ampezzo, Italy, June 26–July 2. Alexander Hollaender, who is president of the International Association of Radiation Research, was instrumental in much of the planning and organization of this

congress. R. B. Setlow served as chairman of one of the special symposia held at the meeting. Division investigators who traveled to Italy from Oak Ridge and presented papers at the congress were:

H. I. Adler	Alexander Hollaender	R. B. Setlow
J. G. Brewen	R. F. Kimball	L. H. Smith
C. C Congdon	R. A. McGrath	G. E. Stapleton
F. J. de Serres	E. F. Oakberg	A. C. Upton
U. H. Ehling	R. A. Popp	R. C. von Borstel
A. H. Haber	M. L. Randolph	H. E. Walburg, Jr.
	Jane K. Setlow	

E. H. Y. Chu and D. L. Lindsley, presently on leave from the Division, also spoke at Cortina. Diana M. Popp attended the meeting. Additional papers by J. G. Brewen, P. Carolyn Gooch, Ellen Mattingly, Paul Nettesheim, and M. L. Simmons were read by title.

Former Division members who presented papers concerning work done during their stay at the Biology Division were Gustavo Cudkowicz, M. B. Lion, and D. C. Swartzendruber (by title).

Most of the investigators who were at Cortina attended other meetings or visited scientific institutions during their stay abroad. Before the Radiation Research congress, E. F. Oakberg, A. C. Upton, and H. E. Walburg, Jr., presented papers at the Colloquium on Radiation and Aging, held in conjunction with the International Congress of Gerontology, at Semmering (Vienna), Austria, June 23–24. R. A. Popp also attended.

After Cortina, J. G. Brewen, Alexander Hollaender, and R. F. Kimball spoke at the Colloquium on Changes in the Cell Cycle Affecting Radiosensitivity at Casaccia (Rome), July 7–8. Dr. Kimball also served as moderator of a session on mutation. Gregorio Olivieri, a recent Biology Division investigator from abroad, presented a paper (coauthored by J. G. Brewen) at the Colloquium. R. A. McGrath and E. F. Oakberg attended.

Titles of papers given at the above-mentioned meetings by the investigators listed (and coauthors when applicable) will be found under **Lectures** (see Contents).

Before going to Cortina, H. I. Adler presented a seminar on "Repair of damage to the cell division mechanism in bacteria" at the Chester Beatty Research Institute in London, and another on "Genetic control of cell division and recovery from radiation damage" at the University of Frankfurt, Frankfurt, Germany.

C. C. Congdon attended the Conference on the Hemopoietic Stem Cell at Hinterzarten, Germany, June 19–21, and presented a paper entitled "Stem cells and lymphatic tissues." Then he went to Bern, Switzerland, June 22–24, where he spoke on "Comparison of existing theories on the function of germinal centers" at the Conference on Germinal Centers of Lymphatic Tissues.

While abroad, F. J. de Serres consulted with colleagues in Bern, Switzerland; Pisa, Italy; and Sheffield, England. He spoke on "The correlation between genetic alteration and complementation pattern among nitrous acid-induced mutants of *Neurospora crassa*" at the Institute of General Microbiology, Bern, and at the University of Sheffield.

U. H. Ehling visited institutions in Frankfurt and Berlin, Germany, during July and gave an invited lecture on "Mutation research in mammals" at the Free University, Berlin.

Before and after the meetings at Cortina and Casaccia, Alexander Hollaender visited laboratories in Italy at Milan, Pavia, Parma, Turin, Padua, and Rome; and in Greece at Athens. While in Athens, Dr. Hollaender and Division members D. L. Lindsley, R. C. von Borstel, and E. F. Oakberg met with members of the organizing committee of a symposium on Genetic Polymorphism to be held in Athens, in cooperation with the Biology Division, in May 1968. The committee members to date are Drs. C. Krimbas, S. Georgopoulos, Ph. Fessas, and C. Christodoulou. A tentative program was drafted. Further details on the program, plans, committee, and sponsors will appear in future semiannuals.

R. F. Kimball visited laboratories in Rome and Naples for discussions with fellow scientists on problems of mutual interest.

In June, R. A. McGrath gave four invited seminars in four countries. He spoke on "Cellular repair mechanisms" at the Free University, Berlin, and at the Centro de Studi Nucleari della Casaccia, Comitato Nazionale per l'Energia Nucleare, Rome; on "Correction on radiation damage" at the University of Grenoble, Grenoble, France; and on "Repair of x-ray damage in *E. coli*" at the Medical-Biological Laboratory RVO-TNO, Rijswijk, The Netherlands.

During visits to Scandinavian laboratories in Norway, Sweden, and Denmark in June, E. F. Oakberg gave invited seminars entitled "Radiation response of spermatogonia in man" at the Norsk Hydro's Institute for Cancer Research, Montebello, Norway, and at the Karolinska Institute, Stockholm.

Before the meetings at Semmering and Cortina, R. A. Popp gave invited lectures in Czechoslovakia and Germany. At the Czechoslovakian Academy of Sciences in Prague he gave a seminar on "Marrow transplantation in mice," and at the Max-Planck Institute for Biochemistry in Munich he spoke on "Hemoglobin variants in mice." After Cortina he presented both of the seminars at the Institute of Genetics in Turin, Italy.

In July M. L. Randolph visited the International Bureau of Weights and Measures in Sèvres, France, where he discussed radiation dosimetry standards with fellow scientists. He then visited the University of Liege, Liege, Belgium, before returning to Oak Ridge.

Jane K. Setlow attended the Conference on Regulatory Mechanisms in Nucleic Acid and Protein Biosynthesis in Lunteren, The Netherlands, June 5–10. During the time between that meeting and the International Congress of Radiation Research at Cortina, she visited several laboratories in The Netherlands and Belgium and gave a seminar "Reactivation of lethal uv damage in vivo and in vitro" at the Medical-Biological Laboratory RVO-TNO, Rijswijk, The Netherlands.

R. B. Setlow presented a paper entitled "Repair of DNA" at the Conference on Regulatory Mechanisms in Nucleic Acid and Protein Biosynthesis, Lunteren, The Netherlands. After visits to laboratories in Delft, Rijswijk (where he lectured on "Repair of uv damage in *E. coli*" at the Medical-Biological Laboratory RVO-TNO), and Utrecht, The Netherlands, and in Brussels, Dr. Setlow returned to the United States to attend the Gordon Research Conference on Nucleic Acids of which he was co-chairman. He then returned to Europe to speak at Cortina.

L. H. Smith attended the Conference on the Recovery of Irradiated Cells by Nucleic Acids at Herceg-Novi, Yugoslavia, June 23–24, and presented a paper entitled "An *in vitro* bone marrow system for studying recovery from radiation injury."

G. E. Stapleton visited institutions in England and The Netherlands before returning to the Laboratory on July 15.

Following the congress at Cortina, R. C. von Borstel visited biological institutes in Greece and Israel to discuss problems of mutual interest with investigators of those countries. In the latter country, he gave seminars on "Super-suppressors" and "Action of DNA and RNA polymerase on fixed cell preparations" at Hebrew University, Jerusalem; on "Super-suppressors" at the Weizmann Institute, Rehovoth; and on "Chromosome breakage and dominant lethality" at the Israel Institute for Biological Research, Ness-Ziona.

H. E. Walburg, Jr., visited the Czechoslovakian Academy of Sciences, Prague, before attending the conferences in Semmering and Cortina.

M. A Bender was in Rio de Janeiro and Guarapari, Brazil, June 2–10, as an advisor to the High Radiation Background Project being carried out by the Institute of Biophysics of the University of Brazil.

Iracema M. Baccarini, a visiting investigator from Brazil during 1965, presented a paper coauthored by A. C. Upton and T. T. Odell, Jr., at the First Pan American Congress of Anatomy in Mexico City, Mexico, July 23–28. The paper was entitled "Histological, histochemical, and autoradiographic studies of the estrous cycle in the mouse." Dr. Baccarini is presently at the Department of Pathology, University of Chicago Medical School, Chicago, Illinois.

Before beginning an eight-month leave of absence from the Biology Division, L. G. Caro visited the Institut de Biologie Physico-Chimique in Paris, May 27–28; spoke on "Principles of high-resolution autoradiography" at the International Symposium on Electron Microscopy and Cytochemistry, Leiden,

The Netherlands, May 30–June 4; and attended the Conference on Regulatory Mechanisms in Nucleic Acid and Protein Synthesis in Lunteren, The Netherlands, June 5–10.

W. E. Cohn, Secretary of the Combined Commission on Biological Nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry, attended a meeting of the commission in Gothenburg, Sweden, July 4–6. He then visited laboratories of the National Institute of Public Health, Oslo, and gave a seminar on "Rapid chromatographic analysis of nucleotide material" at the University of Oslo.

F. J. Finamore went to Windsor, Ontario, Canada, March 31–April 2, to confer with colleagues at the University of Windsor and to present a seminar entitled "Nucleic acid metabolism in normal and x-irradiated brine shrimp embryos."

P. Carolyn Gooch presented an invited paper, "Cytogenetic methods: Somatic chromosome aberrations in occupationally irradiated humans," of which she was coauthor with M. A Bender, at the EURATOM-sponsored Symposium on Accidental Irradiation at Place of Work, held in Nice, France, April 26–29.

M. G. Hanna, Jr., presented two papers at the Conference on Germinal Centers of Lymphatic Tissues, Bern, Switzerland. One, coauthored by Takashi Makinodan and W. D. Fisher, was entitled "Lymphatic tissue localization of ^{125}I-labeled heterologous and isologous macroglobulins"; the other, "Morphologic and autoradiographic studies of spleen white pulp germinal centers during an immune reaction," was coauthored by D. C. Swartzendruber and C. C Congdon. He also attended the Conference on the Hemopoietic Stem Cell, Hinterzarten, Germany, June 19–21.

J. L. Liverman attended the Symposium on the Molecular Biology of Viruses, June 27–30, in Edmonton, Alberta, Canada.

In February, H. V. Malling visited laboratories in England, Sweden, and Denmark for consultations and discussions with fellow-scientists on various aspects of his work.

A paper, "Transformation *in vitro* and solid tumor formation associated with a murine leukemia virus," by R. L. Tyndall, Ernestine Teeter, J. A. Otten, N. D. Bowles, and A. C. Upton, was presented by Dr. Tyndall at the International Congress for Microbiology held in Moscow, July 24–30. After the congress, Dr. Tyndall visited laboratories in London and Manchester, England; Glasgow, Scotland; and Paris to confer with European microbiologists working in his field of interest.

Tuneo Yamada spent the last week in June at the University of Edinburgh (Scotland) as a nonresident fellow and discussed investigations being carried out there. In July, he attended the meeting of the British Society of Developmental Biology, Aberdeen, Scotland, and then went to Wilhelmshaven, West Germany, where he discussed embryonic induction and gave a seminar on "Cellular and subcellular events in Wolffian lens regeneration" at the Max-Planck Institut für Meeresbiologie.

Honors and Appointments

Alexander Hollaender and R. F. Kimball were honored at the Third International Congress of Radiation Research that convened at Cortina d'Ampezzo, Italy, June 26–July 2.

Dr. Hollaender, whose term as President of the International Association of Radiation Research ended at this Congress, was appointed Honorary President of the organization. Dr. Kimball was elected Secretary-Treasurer. (See Foreign Travel, p. 26, for other details of this meeting.)

Meetings and Conferences

TENTATIVE PROGRAM: TWENTIETH ANNUAL BIOLOGY RESEARCH CONFERENCE (1967)

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

SYMPOSIUM ON CHROMOSOME MECHANICS AT THE MOLECULAR LEVEL

Monday, April 10

Morning

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

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

Molecular Aspects of Ring Structures – C. A. Thomas, Jr., The Thomas C. Jenkins Department of Biophysics, The Johns Hopkins University, Baltimore, Maryland

Afternoon

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

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

Replication of the Bacterial Chromosomes – John Cairns, Cold Spring Harbor Laboratory of Quantitative Biology, Cold Spring Harbor, New York

Tuesday, April 11

Morning

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

Regulation of Replication Studies in Bacterial Episomes – Francois Cuzin, Commissariat a l'Energie Atomique, Service de Biochimie, Gif-sur-Yvette, France, and Francois Jacob, Institut Pasteur, Paris, France

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

Afternoon

(Free)

Wednesday, April 12

Morning

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

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

Homologous and Nonhomologous Recognition Between Chromosomes in the Female of Drosophila Melanogaster – Rhoda F. Grell, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Afternoon

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

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

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

Reactions Occurring at Termini in DNA – Jerard Hurwitz, Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York

Thursday, April 13

Morning

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

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

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

PROCEEDINGS OF NINETEENTH ANNUAL BIOLOGY RESEARCH CONFERENCE (1966)

The Proceedings of the Nineteenth Annual Biology Research Conference, **Differentiation and Growth of Hemoglobin- and Immunoglobulin-Synthesizing Cells**, held April 4–7, 1966, in Gatlinburg, Tennessee, were published as a June supplement to the *Journal of Cellular Physiology*.

ANNUAL INFORMATION MEETING SCHEDULED

The Oak Ridge National Laboratory Advisory Committee for Biology will make its annual visit to the Division December 7, 8, and 9. The committee is composed of C. E. Carter, Western Reserve University Medical School; Rollin D. Hotchkiss, Rockefeller University; Henry S. Kaplan, Stanford University Medical School; Herschel Roman, University of Washington; Earl R. Stadtman, National Heart Institute; and Curt Stern, University of California.

During 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 MEETINGS

R. F. Kimball and D. G. Doherty attended meetings of the Biomedical Program Directors of the United States Atomic Energy Commission. Dr. Kimball attended the meeting held at Berkeley, California, February 7–8, and Dr. Doherty attended the meeting held at Idaho Falls, Idaho, May 23–24.

These meetings are held three times each year to review research in the various biomedical programs at Commission laboratories.

BONE MARROW CONFERENCES

April 1966 Conference. – The most recent of the series of regular bone marrow transplantation and chemical radiation protection meetings was held on April 12 at Atlantic City, New Jersey, in conjunction with the meetings of the Federation of American Societies for Experimental Biology. George W. Santos, The Johns Hopkins School of Medicine, served as chairman, and the abstracts of the meeting were published in *Experimental Hematology No. 10.*

June 1966 Conference. – Hans Cottier of the University of Bern was chairman of a conference committee that arranged the program of the International Conference on Germinal Centers of Lymphatic Tissues held in Bern, Switzerland, June 22–24. Included among the more than 53 papers presented under 16 major session topics were three by Division members. These were "Comparison of existing theories on the function of germinal centers" by C. C Congdon and M. G. Hanna, Jr.; "Lymphatic tissue localization of ^{125}I-labeled heterologous and isologous macroglobulins" by M. G. Hanna, Jr., Takashi Makinodan, and W. D. Fisher; and "Morphologic and autoradiographic studies of spleen white pulp germinal centers during an immune reaction" by M. G. Hanna, Jr., D. C. Swartzendruber, and C. C Congdon. Abstracts of many of the papers presented are being printed in *Experimental Hematology No. 11,* and the full texts of all the papers, with summaries of the discussions, will be published.

Future Meetings Scheduled. – The next in the series of regular bone marrow conferences is planned for December 2–3, 1966, at the University of Michigan, Ann Arbor. Byron S. Berlin, School of Public Health, is coordinator of the groups at Ann Arbor that will sponsor the conference.

An evening session for the bone marrow conference group will be arranged for April 17, 1967, at the time of the Federation of American Societies for Experimental Biology meetings in Chicago.

The year 1967 marks the tenth anniversary of the bone marrow conferences and plans are under way to organize a ten-year summary of achievements in this field. The tenth-year conference will be held in Oak Ridge during November or December 1967. Further announcements about the tenth-year winter meeting in Oak Ridge will appear in future issues of *Experimental Hematology.*

Experimental Hematology. – In addition to the abstracts of the April 1966 conference, an invited paper, "Effects of vinblastine on megakaryocytes and platelets of rats," by T. T. Odell, Jr., Marianne Jefferson, C. W. Jackson, and Rebecca S. Reiter, was published in *Experimental Hematology No. 10.*

Contributed papers by W. H. McArthur and by D. B. Thomas and C. C Congdon are included in *Experimental Hematology No. 11.*

THIRD NEUROSPORA CONFERENCE

Approximately 125 representatives from the United States and abroad met in Oak Ridge May 12–14 to attend the Third Neurospora Conference. The meeting was cosponsored by the Biology Division, the National Science Foundation, and the University of Michigan (Ann Arbor).

Division participants were W. E. Barnett, F. J. de Serres, B. J. Kilbey, H. V. Malling, C. E. Terry, and B. B. Webber. Topics of current interest in five major areas were discussed in separate sessions. Presentations were short and informal, with emphasis on general discussion.

Dr. de Serres presented opening remarks at the first session, and Alexander Hollaender gave the welcoming address.

Educational Activities

STUDENT TRAINEE PROGRAM

During this period, 43 undergraduate students spent the summer in the Division.

Thirty-two student trainees carried out research under the direct supervision of a Biology Division senior investigator. Each student presented a final oral and written report of his summer's work.

The 1966 student trainees, their affiliations, and supervisors and groups to which they were assigned were as follows:

STUDENT TRAINEE	INSTITUTION	ASSIGNED TO
David Christian Brehe	Westminster College Salt Lake City, Utah	Ellen Mattingly Rhynchosciara Genetics
Charles Edward Crase	Berea College Berea, Ky.	Lawrence Rosen Enzymology
Hervey Leigh Cunningham	Vanderbilt University Nashville, Tenn.	F. J. de Serres Fungal Genetics
Sheryl Kay Cushenberry	Stephen F. Austin State College Nacogdoches, Tex.	R. B. Cumming Mammalian Genetics
Diane M. Dreher	Rosary Hill College Buffalo, N.Y.	R. K. Fujimura Nucleic Acid Enzymology
Rose Mary Dziak	College Misericordia Dallas, Pa.	W. D. Fisher General Physiology and Radiobiology
Frank M. Eggers II	Maryville College Maryville, Tenn.	G. David Novelli Enzymology
Susan Maria Elsevier	Vanderbilt University Nashville, Tenn.	W. E. Barnett Nucleic Acid Enzymology
Donna Leigh Emerson*	Converse College Spartanburg, S.C.	W. D. Gude Pathology and Physiology
John Edward Geltosky	Memphis State University Memphis, Tenn.	M. P. Stulberg Enzymology
Kathleen Louise Grandi	College of the Holy Names Oakland, Calif.	G. L. Whitson Cytology and Genetics
Terryann Gurney	Erskine College Due West, S.C.	W. D. Wicks, F. T. Kenney Biochemical Regulation
Jane Frances Hable	Alverno College Milwaukee, Wis.	H. I. Adler Radiation Microbiology and Microbial Genetics
Lorraine Mary Kershner	Cedar Crest College Allentown, Pa.	J. G. Brewen Human Cytogenetics
Donald H. Lauer	Indiana University Bloomington, Ind.	R. F. Kimball Cytology and Genetics

STUDENT TRAINEE	INSTITUTION	ASSIGNED TO
Stephen David Leonard	Oberlin College Oberlin, Ohio	B. K. Zimmerman Biophysics
Jane Ann Mays	Oklahoma College of Liberal Arts Chickasha, Okla.	Roy Curtiss III Radiation Microbiology and Microbial Genetics
Lois Kathryn Miller	Upsala College East Orange, N.J.	Peter Pfuderer Enzymology
Georgia Morris	Tennessee Wesleyan College Athens,	Tuneo Yamada Cell Growth and Differentiation
Dianne Mary Murphy	Virginia Polytechnic Institute Blacksburg	G. E. Stapleton General Physiology and Radiobiology
Marjorie Ann Murray	Briar Cliff College Sioux City, Iowa	C. J. Wust Enzymology
John Lee Otis	Kenyon College Gambier, Ohio	J. E. Donnellan, Jr. Biophysics
Paul Dwight Panum	Dana College Blair, Neb.	R. C. von Borstel Cytochemistry and Cell Reproduction
William Henry Ramsey	Dartmouth College Hanover, N.H.	Peter Mazur, S. P. Leibo Cell Physiology
Carolyn Perry Richardson	Randolph-Macon Woman's College Lynchburg, Va.	D. E. Foard Plant Physiology
Sandra Roland	Carson-Newman College Jefferson City, Tenn.	R. A. McGrath Biophysics
Bill Stuart Schnall	Kenyon College Gambier, Ohio	L. H. Smith Mammalian Recovery
Paul Bruce Selby	Westmar College Le Mars, Iowa	Liane B. Russell Mammalian Genetics
Susan Louise Walker	University of Pennsylvania Philadelphia	R. A. Wallace Cell Growth and Differentiation
Mary Susanna Wedemeyer	Creighton University Omaha, Neb.	C. C Congdon Mammalian Recovery
Vivien Brewer Whitfield*	University of Tennessee Knoxville	Ellen Mattingly Rhynchosciara Genetics
Willian Duncan Willis	Pfeiffer College Misenheimer, N.C.	A. H. Haber Plant Physiology

*Will return to the University of Florida, Gainesville.

OTHER STUDENTS

The following undergraduate students also worked in the Division this summer:

Robert Dennis Batey	Tennessee Agricultural and Industrial State University Nashville	Experimental Animal Facility
Patricia Gail Carver	University of Tennessee Knoxville	Administrative Office
Mary Ann Childs	University of Tennessee Knoxville	Administrative Office
Thomas Eugene Banker	University of Tennessee Knoxville	Inhalation Carcinogenesis
Donald Craig Brater	Duke University Durham, N.C.	Molecular Anatomy section
Patricia Frances Davis	Sweet Briar College Sweet Briar, Va.	Cell Physiology
Gary Steven Reid	Tennessee Agricultural and Industrial State University Nashville	Experimental Animal Facility
Katherine Elner Settles	Knoxville College Knoxville, Tenn.	Media kitchen
Katherine Anne Smith	Tennessee Technological University Cookeville	Administrative Office
Karen Lee Johnson	University of Tennessee Knoxville	Administrative Office
Sheron Gale Armes	Berea College Berea, Ky.	Media kitchen

SENIOR TRAINEES

The following students, who have completed work for their B.S. degrees and will be in graduate school this fall, spent the summer in the Division in the groups indicated:

SENIOR TRAINEE	INSTITUTION	ASSIGNMENT
Alan Bachrach, Jr.	University of Pennsylvania School of Veterinary Medicine Philadelphia	Experimental Animal Facility
William Pressly Hunt	Emory University School of Medicine Atlanta, Ga.	Enzymology
Louis Victor Kaufman	West Virginia University School of Medicine Morgantown, W.Va.	Enzymology
Carol Minette Newman	Florida State University Tallahassee	General Physiology

SENIOR TRAINEE	INSTITUTION	ASSIGNMENT
William Robert Somers	Duke University Durham, N.C.	Radiation Immunology
Ina Kay Wachsmuth	University of Miami Miami, Fla.	Enzymology

Sandra F. Bowles, a graduate student from McGill University, Montreal, Canada, spent the summer working in the laboratory of G. L. Whitson of the Cytology and Genetics section.

UNIVERSITY COOPERATIVE PROGRAM

Ford Foundation Program – 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 ORNL–University of Tennessee Cooperative Research Program sponsored in part by the Ford Foundation.

During the academic year, Dr. Adler has been lecturing on bacterial genetics for the University's Department of Microbiology. Dr. Haber taught a spring-quarter graduate-level course on Special Problems in Botany in the University's Botany Department.

Ellen Mattingly also taught a course on Cytogenetics during the spring quarter in the University's Botany Department under the joint ORNL-UT research program.

Other University Courses Taught – The following courses were taught in cooperation with the University of Tennessee during the spring quarter:

Animal Cytology – Zoology-4310 (Animal Cytology), a graduate-level course that covered structure and composition of major nuclear and extranuclear organelles and relation of structure to function, was taught by O. L. Miller, Jr., at the ORINS-ORAU Special Training Building. Barbara R. Beatty assisted in the course.

Histology – W. D. Gude taught a course on Histology at the ORINS-ORAU Special Training Building.

Human Genetics – J. D. Regan taught a course on Human Genetics for the Department of Zoology of the University of Tennessee in Knoxville.

Modern Zoology – The Modern Zoology course, organized by the Division's G. L. Whitson and N. G. Anderson, was taught at the ORINS-ORAU Special Training Building. It was the third in a series of related courses offered this year for college graduates in nonbiological fields and with no previous courses in zoology.

Biology Division members who have given lectures and conducted laboratory sessions thus far are N. G. Anderson, W. A. Arnold, W. E. Barnett, Roy Curtiss III, D. G. Doherty, L. H. Elrod, F. J. Finamore, W. D. Fisher, Tom Grizzard, W. D. Gude, T. R. Manney, Peter Mazur, R. A. Popp, R. B. Setlow, A. C. Upton, Mayo Uziel, R. C. von Borstel, and G. L. Whitson.

Molecular Biology – W. E. Barnett and Jane K. Setlow taught a course on Molecular Biology at the ORINS-ORAU Special Training Building. The course was the third in a series taught by Dr. Barnett for the University of Tennessee this year. The course covered molecular aspects of gene action, including protein and nucleic acid biosynthesis and their regulatory mechanisms. Also covered were biophysical approaches to investigating these phenomena.

Topics in Biophysics – A course entitled Topics in Biophysics was taught at the University of Tennessee in Knoxville by Drs. R. B. Setlow, M. L. Randolph, J. R. Einstein, and J. E. Donnellan of the Biology Division, and by Dr. D. Q. Brown of the University. It covered physical and mathematical approaches to an understanding of biological structure and function, including x-ray diffraction and crystallography of biological materials, biophysics of muscular contraction and membrane phenomena, molecular photobiology, electron spin resonance spectroscopy in biology, and advanced microscopy.

Participation in Other Courses – W. E. Barnett presented a series of three lectures on "The roles of tRNA in protein synthesis," February 10–12, as part of a nucleic acids course taught at the University of Georgia, Athens.

G. David Novelli presented a series of three lectures on "Mechanisms in the biosynthesis of proteins," as part of a biochemistry course offered at the University of Georgia, Athens, March 7–9.

PARTICIPATION IN INSTITUTES, SPECIAL COURSES

W. D. Gude spoke on "Autoradiography," February 17, as part of the Medical Radioisotope Course offered at the ORINS-ORAU Medical Division.

Eight Division scientists spoke during the ORINS-UT-AEC Radiobotany Institute, June 13–August 5, at the ORINS-ORAU Special Training Building. They were: H. I. Adler, "Genetic control of radiation sensitivity in bacteria"; J. G. Brewen, "Kinetics of induced chromosome aberrations"; D. E. Foard, "The use of γ plantlets in studies of growth and development"; A. H. Haber, "Radiation effects on germination"; H. E. Luippold, "Cytogenetic effects of radiations on *Vicia faba*"; Ellen Mattingly, "Use of radioisotopes in cytology" and "Educational activities at the Biology Division"; M. L. Randolph, "Formation, detection, and consequences of radiation-induced free radicals in biological material"; and G. E. Stapleton, "Types of radiation in biological research." D. G. Gosslee, Biometrics section of the ORNL Mathematics Division, spoke on "Biological statistics."

R. A. Wallace lectured on the subject of "Vitellogenesis" in the Embryology Training Program, Woods Hole, Massachusetts, on June 22.

J. L. Epler presented a lecture on "Protein synthesis" at the National Science Foundation Summer Institute, Le Moyne College, Memphis, Tennessee, on July 15.

Six Division staff members lectured July 18 at the Sixth Annual United States Army Nuclear Science Seminar held in Oak Ridge. Speakers were N. G. Anderson, "Research on liquid centrifuge"; N. K. Clapp, "Chemical carcinogenesis with nitrosamines"; E. B. Darden, Jr., "Effect of radiation on biological organisms"; D. E. Foard, "Radiation studies in plant physiology"; W. D. Gude, "Current research in the Pathology and Physiology section of the Biology Division, ORNL"; and L. J. Serrano, "Controlling variables for a radiobiology experiment on effects of low-level irradiation."

John Papaconstantinou spoke on "Molecular aspects of lens cell differentiation" as part of an embryology course at Woods Hole Marine Biological Station, Woods Hole, Massachusetts, on July 27.

S. P. Leibo attended a course on Bacterial Viruses at Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, July 8–31.

Division Members Working in Other Laboratories

LEAVE OF ABSENCE

W. F. Bertsch is at Cambridge University, England, where he is working in the Biochemistry Department with Dr. Robert Hill. Dr. Bertsch's one-year stay in Cambridge is being jointly supported by a National Institutes of Health Special Fellowship and by ORNL. He is scheduled to return to the Laboratory in late October 1966.

L. G. Caro is working at the Microbial Genetics Research Unit, Medical Research Council, Hammersmith Hospital, London, England, under an eight-month Senior Postdoctoral Fellowship granted by the National Science Foundation. Dr. Caro will return in February 1967.

E. H. Y. Chu is working with Dr. George Klein at the Karolinska Institute, Stockholm, Sweden. Dr. Chu's research in Sweden is being jointly sponsored by ORNL and a National Science Foundation Senior Postdoctoral Fellowship. He will return to the Laboratory at the end of August 1966.

Joan Wright Goodman is on leave of absence to spend six months at the Weizmann Institute of Science, Rehovoth, Israel, under the sponsorship of ORNL and a Louis Lipsky Fellowship. Dr. Goodman will return to the Division in late August 1966.

V. K. Jenkins has been awarded a U.S. Public Health Service Predoctoral Fellowship to carry on graduate study at the University of Tennessee. He is on leave from the Pathology and Physiology group while continuing his study under this fellowship.

D. L. Lindsley is spending a year in Rome, Italy, at the Istituto di Genetica in the laboratory of Professor Giuseppi Montalenti, under the joint sponsorship of a National Science Foundation Senior Postdoctoral Fellowship and ORNL. Dr. Lindsley will return to the Laboratory in September 1966.

O. L. Miller, Jr., is on a three-month leave of absence with the Biochemistry Department at the Centro de Investigación y de Estudios Avanzados, Instituto Politecnico Nacional, Mexico City, Mexico. Dr. Miller is teaching a course on electron microscopy. He is to return to the Laboratory in September 1966.

COLLABORATIVE RESEARCH

J. F. Albright is spending three months at the Jackson Laboratory, Bar Harbor, Maine, to carry on collaborative research with Dr. Elizabeth Russell. Dr. Albright will return to the Laboratory in September 1966.

Cytology and Genetics

R. F. Kimball[a]

Effects of Radiation on Paramecium (1.1–1.6)

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

Phage Genetics (1.7–1.12)

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

Mammalian Genetic Cytology (1.13–1.15)

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

Mammalian Biochemical Genetics (1.16, 1.17)

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

Rhynchosciara Genetics (1.18–1.23)

Crodowaldo Pavan[d]
M. Ellen Mattingly
Günter Weirich[d]
Shirley P. Ogle
C. L. Parker
D. C. Brehe[c]
A. L. P. Perondini[d]
L. C. G. Simões[d]
Vivien B. Whitfield[c]

Participation in Cooperative Programs:

AEC-NIH Chemical Carcinogenesis (22.8–22.10)

[a]Dual assignments.
[b]Loanee.
[c]Student trainee.
[d]Visiting investigator from abroad.
[e]Leave of absence to Karolinska Institute, Stockholm.
[f]USPHS Postdoctoral Fellow.

1.1 TIME OF PHOTOREACTIVATION AND MUTATION YIELD

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

In Sect. 1.2, it is shown that ultraviolet-induced thymine dimers can be detected and measured in *Paramecium aurelia*. Therefore, it seemed desirable to extend our earlier studies on the induction of mutations by ultraviolet light to provide a broader base for comparison between the mutagenic effect and the chemically detected alteration of DNA. Our earlier work[1] had shown that mutation yield could be reduced by photoreactivation, but neither the maximum amount of photoreactivation nor the time range within which it was effective had been determined.

Methods. – Synchronized groups of paramecia were exposed in small quartz tubes to 2537-A ultraviolet light from a 15-w G.E. germicidal lamp. Immediately after irradiation the contents of these quartz tubes were expelled into small petri dishes, which were then either left in the dark or exposed to the light from a pair of 15-w G.E. black lights (major output at 3650 A). Mutation yield was scored in the usual way.

Results. – The results showed that 300,000 ergs/mm^2 incident exposure of the black light was sufficient to reduce the mutation yield to nearly zero for incident doses of 2537-A ultraviolet light ranging from 500 to 1000 ergs/mm^2. This very-high-level photoreactivation was not expected but has been repeated now several times.

Figure 1.1.1 shows the effect of increasing the time between the exposures to 2537- and 3650-A light. The paramecia were irradiated with 2537-A ultraviolet light 30 min after division and then exposed to the black light for 20 min, starting at the times shown. The results suggest an exponential decay of photoreactivability with a half-time of about 2 hr.

Discussion. – Photoreactivation in *P. aurelia* has not yet been shown to be the result of splitting of pyrimidine dimers, but if mutations arise from dimers that have persisted until the time of DNA synthesis, then it would have been expected that maximum photoreactivation should have remained possible until DNA synthesis. In the present material, normal DNA synthesis could not possibly have occurred until 100 min after the exposure to 2537-A light and probably occurred much later because of the induced delay. Indeed it is possible that normal synthesis did not occur until after the longest interval of 440 min. Thus, for mutation, photoreactivability must decay in the absence of normal DNA synthesis. This suggests that the initial pyrimidine dimers are converted into another and nonphotoreactivable form before DNA synthesis. This other form might be either the final mutation itself or some intermediate reversible form. Tests for the latter will be made using post-treatments known to influence dark repair.

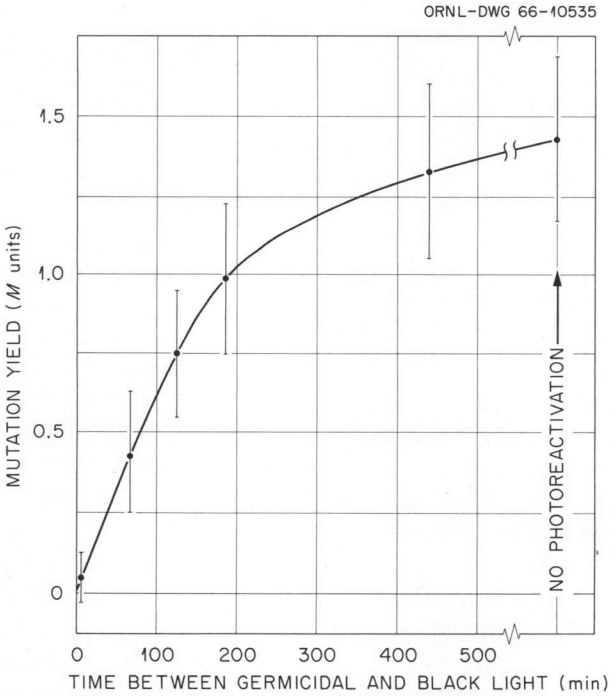

Fig. 1.1.1. Relation Between Mutation Yield and Time Between Exposure to the Germicidal Lamp and the Start of the Exposure to the Black Light. 95% confidence limits are shown.

References

[1]R. F. Kimball and Nenita Gaither, *J. Cell. Comp. Physiol.* **37**, 211–33 (1951).

1.2 PYRIMIDINE DIMERS IN *PARAMECIUM*

Betsy M. Sutherland W. L. Carrier[1]

Ultraviolet light produces pyrimidine dimers in DNA in vivo and in vitro. Several studies have indicated that these dimers are important in the lethal effects of uv; however, there is no clear evidence implicating pyrimidine dimers in uv mutagenesis. To examine the role of pyrimidine dimers in uv-induced mutations, we are investigating the relationship between genetic mutation and dimer production and disappearance in *Paramecium aurelia*, where there is much information about the role of premutational damage in mutation induction.

Berger and Kimball's method[2] of labeling paramecia with tritiated thymidine by way of the food organism *Escherichia coli* has been adapted for mass cultures and high total activity yield, increasing the total activity of the sample by a factor of 10. Autoradiograms of animals labeled by this method showed that the nuclei were heavily labeled, while the cytoplasm was only lightly labeled.

Mass cultures of paramecia examined immediately after uv irradiation at incident exposure of 100,000 ergs/mm^2 or less showed no cell lysis. Assays for pyrimidine dimers[3] in cells irradiated in this exposure range showed that dimers were produced at detectable levels and that the dimer content increased with increasing exposure.

Work is continuing on the level of dimers present immediately after irradiation as well as after post-uv treatment with photoreactivating light and after post-uv growth in the dark.

References

[1]Biophysics section.

[2]J. D. Berger and R. F. Kimball, *J. Protozool.* **11**(4), 534–37 (1964).

[3]K. C. Smith, *Photochem. Photobiol.* **2**, 503 (1963).

1.3 CONTINUATION OF THE STUDIES ON INTRACLONAL VARIATIONS IN CELL MASS AND GROWTH RATE IN *PARAMECIUM AURELIA*

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

Introduction and Methods. – Previous studies[1] have shown that there is considerable variation in dry mass and DNA content within genetically uniform material in *P. aurelia*. These variations persist through a number of cell generations and are apparently the result of changes in growth rate resulting from changes in DNA content resulting in turn from unequal division of the macronucleus.

The previous studies were made mainly by correlation analysis between different lines of descent. To get a better picture of the rate of rerandomization of size differences, the following experiment was carried out. Two dividing paramecia, judged by eye to be of different sizes, were isolated and allowed to divide four more times to produce 32 daughter cells each. Thirty of these 32 were isolated as progenitors of 30 daily single-cell isolation lines. On each of a number of successive days, one recently divided specimen was dried down from each line of descent. The dry mass of each specimen was determined by microinterferometry and the DNA content by microspectrophotometry.

Results. – The mean differences in dry mass between the two groups of 30 lines, one descended from an initially large and one from an initially small specimen, gradually decreased with a half-time of about 12 days. The 30 lines were initially more alike than are lines taken at random, but variability was reestablished rapidly. This new variability, itself, tended to persist once established. That is, the larger sublines in a group of 30 tended to remain larger; the smaller, smaller. These changes in dry mass were closely paralleled by corresponding changes in DNA content.

Discussion. – These results show that differences in lines of descent in dry mass within a clone persist for appreciable periods but that gradual randomization occurs. They also show that such persistent differences are continuously arising within a line of descent. Finally, they confirm the close correlation between dry mass and DNA content.

References

[1]R. F. Kimball, Stella W. Perdue, and E. G. Bailiff, *Biol. Div. Semiann. Progr. Rept. Feb. 15, 1965*, ORNL-3768, p. 36; *July 31, 1965*, ORNL-3853, pp. 36–37; and *Jan. 31, 1966*, ORNL-3922, pp. 33–34.

1.4 PRODUCTION OF THYMINE DIMERS IN ULTRAVIOLET-IRRADIATED *TETRAHYMENA*

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

Introduction. – It has been suggested from observations on the delaying effects of low doses of ultraviolet light on cell division in *Tetrahymena pyriformis* that some ultraviolet-sensitive substance interacts with the so-called "division protein" in these cells.[2] Although there have been several reports concerning delays, photoreactivation, and lethality of different doses of ultraviolet light on cell division in *Tetrahymena*,[3–5] no ultraviolet-induced biochemical products have been isolated or identified in this organism. With this in mind, we decided to first look for thymine dimers in log-phase cultures of *Tetrahymena*.

Methods and Results. – Log-phase cells grown in 2% proteose peptone with 0.4% liver extract and salts were washed and resuspended in inorganic salt medium. Different samples of these cells were then given doses of 500 and 2000 ergs/mm^2 of 2652-A ultraviolet light. We have

qualitatively identified thymine dimers in hydrolysates of cells given both of these doses of ultraviolet light. We are now in the process of looking at survival from different doses of ultraviolet light, for photoreactivation, and dark repair in this organism.

References

[1]Biophysics section.

[2]D. S. Nachtwey and A. C. Giese (unpublished results).

[3]D. S. Nachtwey, *Dissertation Abstr.* **22**, 1758 (1961).

[4]R. M. Iverson and A. C. Giese, *Exptl. Cell Res.* **13**, 213 (1957).

[5]J. Calkins, *Nature* **196**, 686 (1962).

1.5 CHANGES IN SOLUBLE CARBOHYDRATES OF LOG-PHASE CULTURES AND SYNCHRONIZED *TETRAHYMENA*

G. L. Whitson J. G. Green[1]

Studies are being continued on the changes in soluble carbohydrates of log-phase and synchronized *Tetrahymena* by the use of a prototype carbohydrate analyzer which permits fractionation of sugars from cells with a single operation.[2] The cells were grown on 1% proteose peptone containing 0.1% liver extract. Synchronized cells[3] were sampled at the end of the last heat shock, just prior to cell division, during the peak of cell division, and after cell division had occurred. Cells were pelleted by centrifugation, and 1-ml aliquots of packed cells were lysed with saturated indole contained in Tris buffer pH 7.0. The soluble sugars were then extracted with cold 80% ethanol, and the supernatant was concentrated by flash evaporation at room temperature. Two milliliters of borate elution buffer was then added, and 1 ml of each sample was analyzed for the soluble sugar content. Fourteen soluble carbohydrate compounds appear in the cell extracts during the predivision period. The concentrations of some of these compounds vary cyclically in relation to cell division. Although it appears that many of these compounds are metabolized during cell division, the significance of these changes remains to be assessed. A number of components of log-phase cells have been identified, including 2-D-deoxyribose, ribose, mannose, fructose, arabinose, xylose, and glucose. Additional compounds also occur which have not yet been identified.

References

[1]AEC-NIH Molecular Anatomy Program.

[2]J. G. Green, *Natl. Cancer Inst. Monograph* **21**, 447 (1966).

[3]O. H. Scherbaum and E. Zeuthen, *Exptl. Cell Res.* **6**, 221 (1954).

1.6 RECOVERY FROM RADIATION-INDUCED DIVISION DELAY AND GIANTISM IN *SPATHIDIUM SPATHULA*

D. B. Williams

Spathidium spathula is highly sensitive, for a ciliate, to x rays,[1] responding unusually by showing division delay and giantism. The injury is temporary at low doses (4 to 6 kr) but is permanent at higher doses (25 to 55 kr). This report is concerned with the low-dose effect. The process of recovery of the normal generation time and cell mass is described. Spathidia were exposed to Mitomycin C and triethylene melamine, agents which degrade DNA, to test the possibility that the primary site of radiation damage is DNA in the macronucleus. The general conclusions are: (1) Spathidia irradiated with 5 kr of x rays have a first generation time more than twice the normal length. (2) During the extended first generation, cell growth (dry mass) continues at nearly the normal rate to produce cells that are two or more times the normal mass at division. (3) The second generation time is significantly shorter (about 35%) than normal. (4) The average dry mass at division 2 is about 1.4 times the normal amount. (5) The third generation is also significantly shorter than normal, and the normal generation time is recovered by the fourth division. (6) The longer a cell is delayed from dividing in the first generation, the greater its dry mass and the shorter the second generation. (7) Cell division normally results in the protein having a larger cell mass (6 to 14%) than the opisthe, and x irradiation exaggerates the difference. (8) Mitomycin C is effective at

low doses in producing giantism and permanent injury, but the full effect is not expressed until two or more generations. (9) Mitomycin C giant cells have considerably more than the normal amount of macronuclear material, suggesting continued DNA synthesis, but the large number of nuclear fragments in the cytoplasm also indicate macronuclear degradation. (10) Total micronuclear loss may also follow exposure to Mitomycin C. (11) Triethylene melamine is also effective in producing giantism and micronuclear loss. (12) DNA synthesis continues to occur in TEM division-delayed giants, as shown by the massive amount of macronuclear material and a 70- to 80-fold increase in the number of micronuclei in some cells three days after exposure. (13) Spathidia respond to ultraviolet (2537 A) in a manner analogous to that found for x rays. The first generation is lengthened and the cells become larger than normal. The second and third generations are shorter than normal, and the normal generation time is expressed by the fourth division after exposure to 2000 ergs/mm^2. (14) *Spathidium* is about three times more resistant to division delay, induced by exposure to 500–2000 ergs/mm^2 of 2537-A ultraviolet, than *Paramecium aurelia.*

Agents which degrade and interfere with DNA synthesis mimic the x-ray response displayed by *Spathidium,* whereas inhibitors of protein and RNA synthesis[2] do not. These results support the interpretation that the unusual radiosensitivity shown by *Spathidium* may result primarily from specific damage to DNA in the macronucleus.

References

[1]D. B. Williams, *J. Protozool.* **9**, 119–22 (1962).
[2]D. B. Williams, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1966,* ORNL-3922, pp. 34–35.

1.7 ACTION SPECTRA FOR ULTRAVIOLET-INDUCED MUTATION IN *ESCHERICHIA COLI*

Jane K. Setlow M. E. Boling

Introduction. – It has been postulated that some of the premutational lesions which could lead to reversion to prototrophy are normally repaired relatively slowly after the irradiated cells have been plated. Another fraction of lesions is apparently repaired very rapidly. The addition of acriflavin to the plating medium, which drastically inhibits repair, should select a particular class of mutations which result from inability to carry out the slower repair of premutational lesions on the plates. If this class of mutations is caused by a different photochemical lesion from the one which remains unrepaired on normal plates, the action spectrum for mutation should be different when the plating medium contains acriflavin. Furthermore, we might expect a strain which is poor in repair of some premutational lesions to have an action spectrum for mutation similar to that of the strain with normal repair plated with acriflavin.

Results and Discussion. – The strain with normal repair has an entirely different action spectrum for mutation when acriflavin has been added to the plating medium. This spectrum resembles the spectrum for killing (probably mostly the result of pyrimidine dimers), whereas the normal mutation spectrum does not. The strain poor in repair has an action spectrum for mutation with and without acriflavin which resembles the acriflavin spectrum of the other strain. These results suggest that there are at least two possible premutational lesions for prototrophy reversion, a dimer and another unknown lesion, and that the premutational dimers are only important when repair is impeded, either because of a mutation or because of an inhibiting chemical.

1.8 PHOTOREACTIVATING ENZYME IN YEAST

M. E. Boling Jane K. Setlow

Introduction. – A study of photoreactivating enzyme levels in various strains of yeast grown in different ways was undertaken with the idea that possibly the enzyme has some other function in the cell besides splitting pyrimidine dimers in uv-irradiated yeast and that such information might provide a clue to the nature of the hypothetical second function.

Results and Discussion. – The criterion for enzyme level was the rate at which extracts of the cells at a fixed protein concentration were able to increase the biological activity of uv-irradiated transforming DNA from *Hemophilus influenzae.* There were no significant differences found between related haploid and diploid strains, between

cells grown aerobically or anaerobically, or between cytoplasmic petite strains and their related normal strains. However, a big difference was found between enzyme levels of logarithmically growing and stationary cells of the same strain. In the case of four different strains, the stationary cells contained 6 to 25 times more enzyme than the log cells. This difference is in accord with the difference in the rate of photoreactivation of whole cells at 4047 A, namely, very slow photoreactivation in logarithmically growing cells and rapid photoreactivation in stationary cells. It is concluded that in logarithmically growing cells photoreactivating enzyme is either in short supply or is somehow tied up and unavailable for reaction with the yeast DNA in whole cells or with transforming DNA in cell extracts.

1.9 PHOTOREACTIVATING ENZYME IN *NEUROSPORA*

C. E. Terry[1] Jane K. Setlow

Introduction. – Extracts of *Neurospora crassa* contain photoreactivating enzyme, by the criterion of ability of the extracts to increase the transforming ability of uv-irradiated *Hemophilus influenzae* DNA. We have found that such activity is heat labile and is destroyed by trypsin. The wavelength dependence of such in vitro photoreactivation has been studied for the purpose of (1) characterizing the enzyme and (2) comparison with the in vivo action spectrum for photoreactivation in *Neurospora*.

Results and Discussion. – The action spectrum is a simple one, with a single maximum at 4047 A in the range 3132 to 4358 A, differing from the more complicated in vitro spectra for yeast and *Escherichia coli*. However, the in vitro *Neurospora* spectrum coincides closely with the in vivo spectrum for this organism. This suggests that there is little or no "indirect" photoreactivation in *Neurospora*. It is concluded that the *Neurospora* photoreactivating enzyme is probably of a different type from that of yeast and *E. coli*.

References

[1]Radiation Microbiology and Microbial Genetics section.

1.10 PURIFICATION OF THE YEAST PHOTOREACTIVATING ENZYME

Amir Muhammed Dianne C. Brown

The photoreactivating enzyme has been purified on a large scale from bakers' yeast using $(NH_4)_2SO_4$ precipitation, batchwise absorption and elution from phosphocellulose, and column chromatography on phosphocellulose and hydroxylapatite. A final purification of 5000- to 6000-fold over the crude extract has been achieved. The purified enzyme has a characteristic protein absorption spectrum with a sharp peak at 279 mμ and very little absorption from 300 to 520 mμ, where it is known that absorption for photoreactivation takes place. The enzyme has been completely freed from traces of cytochrome without loss of activity, and thus cytochrome has been eliminated as a possible chromophore in the reaction. The enzyme behaves as a homogeneous preparation on ultracentrifugation and Sephadex gel filtration.

When a mixture of uv-irradiated DNA and the purified enzyme layered on 10% sucrose is centrifuged at 36,000 rpm, the enzyme activity disappears from the top of the tube, whereas unirradiated DNA does not carry down the activity. However, very little protein is removed from the top of the tube along with the irradiated DNA, suggesting that the purified enzyme preparation contains more than 90% inactive protein molecules that do not bind to the substrate. This hypothesis was also confirmed by passing the enzyme and uv-irradiated DNA through a DEAE-cellulose column. The purified enzyme alone does not bind to this substance, and all the activity can be recovered in the filtrate. However, when the enzyme is first mixed with irradiated DNA, DNA binds to the DEAE and the enzyme activity does not pass through, although most of the protein does. Around 25% of the activity bound to the DNA can be eluted with 0.5 *M* NaCl at pH 6.5. Experiments are in progress to attempt to recover the enzyme without loss of activity.

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

C. M. Steinberg R. C. von Borstel
H. R. Gwinn[1] E. W. McDaniel[1]
S. A. Reynolds[2]

We have repeated our measurement of the cross section of ^{33}S to thermal neutrons. The conditions

of irradiation were the same as before,[3] except that larger samples (10 mg) and a somewhat longer exposure time (4.5 days) were used in order to improve accuracy. Estimates of the cross section from two samples (irradiated at two different positions in the Argonne CP-5 reactor) are 4.4 and 4.6 mb. These values are in essential agreement with our earlier (less accurate) estimate of 6 (±1) mb; they do not agree with values (2.2 and 2.3) obtained by Reynolds and Lewis[4] using somewhat different techniques. The reason for the disagreement is not known.

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1.12 A SEARCH FOR PHOTOREACTIVATING ENZYMES IN *DROSOPHILA MELANOGASTER* EXTRACTS

Amir Muhammed J. E. Trosko

Photoreactivating (PR) light given as a post-treatment increases embryo survival and adult fertility and decreases recessive lethal mutation rate after low doses of ultraviolet (uv) radiation to polar cap cells of *Drosophila*.[1] Also, uv-induced phenocopies in pupae can be photoreactivated. The question of whether these phenomena were dependent on a photoreactivating enzyme similar to that in yeast and *Escherichia coli* was investigated. Eggs, larvae, pupae, and adult *Drosophila* were grown aseptically and homogenized in cold 0.05 *M* phosphate buffer (pH 7.3). The homogenates were sonicated for 20 sec to rupture cells and were then filtered through Miracloth to remove chitinous debris.

The photoreactivating enzyme activity of different extracts was tested by their ability to photoreactivate uv-irradiated transforming DNA. Calf thymus DNA was added to the reaction mixtures in order to protect the transforming DNA against DNase. The results of a typical experiment are summarized in Table 1.12.1.

No photoreactivating enzyme activity was detected under the assay conditions used in these experiments. It was realized that the lack of PR activity in the extracts might be due to the presence of some substrates which could strongly inhibit the enzyme activity. When purified yeast PR enzyme was added to the extract, however, there was no loss in activity, indicating the absence of any inhibitory material in *Drosophila* extracts. The extracts also did not photodynamically inactivate added transforming DNA. This suggests that the reported photoreactivation phenomena in *Drosophila* are mediated through an indirect effect.[2] Another possibility is that the photoreactivating enzyme is present in very small amounts, or that the enzyme in *Drosophila* is of a different type than the one in microorganisms, and is not detectable by the assay used in this work.

Table 1.12.1. Photoreactivating Enzyme Activity of Various Extracts from *Drosophila*

Extract	Number of Streptomycin Transformants		Ratio of PR/Dark Transformants
	Dark Control	PR	
Growth medium	1081	1065	0.99
Eggs	1201	1220	1.02
Larvae, three-day	1112	1184	1.06
Larvae, seven-day	930	899	0.97
Pupae, one-day	1093	1129	1.03
Pupae, two-day	1109	1110	1.00
Adult	1373	1323	0.96

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1.13 STUDY OF PYRIMIDINE DIMERS IN MAMMALIAN CELLS SURVIVING LOW DOSES OF ULTRAVIOLET RADIATION

J. E. Trosko Margaret R. Kasschau

Many bacterial strains are resistant to killing by ultraviolet radiation, and such strains have the ability to excise ultraviolet-induced pyrimidine dimers from their DNA. However, it has been shown that pyrimidine dimers are not excised as parts of small TCA-soluble pieces in certain strains of mammalian cells grown in vitro. The latter studies were carried out at high doses and left the following questions unanswered: (*a*) Does excision occur at low doses where an appreciable fraction of the cells survive? (*b*) Could excision have occurred in an oligonucleotide and hence have been undetected? (*c*) Could excision occur if the cells have a longer time to repair the damage? To answer these questions, the ^{3}H-Tdr-labeled nuclear material of ultraviolet-irradiated (35 ergs/mm^2) Chinese hamster cells was separated by Sephadex G-100 column chromatography into three fractions [large polynucleotides, oligonucleotides, and small oligo- and mononucleotides (<5 nucleotides and various small thymidine-derivative molecules)]. Each fraction was analyzed for dimers. It was found that, in this strain of mammalian cells, the pyrimidine dimers remained associated with the large polynucleotide fraction (>20 nucleotides) for at least 48 hr. In this time and at this dose, cells could have divided at least once. Therefore, it is likely that pyrimidine dimers were not removed from the chromosomal DNA and that these cells could replicate DNA with pyrimidine dimers in the priming strands, albeit the new DNA is probably abnormal.

1.14 REDUCTION OF CHROMOSOME BREAKS BY CYSTEAMINE IN 5-BROMODEOXYURIDINE-SUBSTITUTED MAMMALIAN CELLS

J. E. Trosko J. G. Brewen[1]

When 5-bromodeoxyuridine (BUDR) is incorporated into the DNA of mammalian cells, the cells are sensitized to uv radiation with regard to cell killing and chromosome aberrations. Two general mechanisms of sensitization have been postulated: (1) that there are qualitative and quantitative differences in lesions induced in BU-substituted DNA as compared to normal DNA, or (2) BU lesions inhibit normal repair processes. Cysteamine has been shown to negate BU-sensitized killing in T_1 phage and molecular weight reduction in uv-irradiated phage DNA.

Our studies have shown that when cysteamine is added during uv irradiation of BU-substituted mammalian cells, the yield of chromosome breaks is three to four times lower than the yield of nontreated or post-cysteamine-treated BU cells. The reduction of breaks is not due to reduction of the uv incident energy on the chromosome by cysteamine absorption, since pyrimidine dimer formation, a measure of the uv light absorbed by the DNA, is essentially identical in nontreated and treated cells. Also, DNA synthesis is not enhanced in cysteamine-treated cells. Since there is no evidence of a uv-repair system similar to the bacterial dark-repair system and since posttreatment with cysteamine did not reduce the yield of chromosome breaks, it is doubtful that the reduction of uv-induced chromosome breaks is due to enhancement of a repair system.

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1.15 CHARACTERISTICS OF RADIORESISTANT HUMAN AMNION CELLS

J. D. Regan

In a previous report[1] we described the development of an x-ray-resistant human cell line, RA X 10, derived from RA,[2] an established cell line having a triploid stem cell.

Comparative studies on the biology of RA (the control parental line), RA X 10 in early passages, and RA X 10-25 (the radioresistant cell after 25 passages) have been made. Present results are shown in Table 1.15.1.

These data seem to indicate that, following irradiation, an x-ray-resistant, slow-growing cell population appears. These cells have a rather broad distribution of chromosomal modes in a relatively low range for established human cell

Table 1.15.1. Characteristics of Control and Radioresistant Human Amnion Cells

Characteristic	Cell Line		
	RA	RA X 10-12	RA X 10-25
Chromosomal mode	68	Polymodal – broad distribution in numbers <63	66
Generation time, hr	30	>60	30
Plating efficiency, %	60	17	60–70
Fastidiousness, pH sensitivity, etc.	Low	High	Low
X-ray resistance, in relative units	1	1.6–2.0	1.6–1.4
Uv resistance, in relative units	1	No data	2.0–3.0

lines. In addition, these cells are very fastidious in culture and are rather unstable. They give rise to variants which are less radioresistant but more vigorous according to other criteria such as plating efficiency and generation time. The fact that the variants show increased growth rate means that they will eventually overgrow and thus eliminate the resistant cells. Decline of x-ray resistance with increasing passage number has been characteristic of our RA X 10 cultures.

RA X 10 displays elevated survival after ultraviolet irradiation. This survival rate has appeared to be relatively stable in six successive passages since RA X 10-25 (25th passage). We are now therefore studying the effects of ultraviolet rays not only on survival per se but also on DNA synthesis, mitotic inhibition, and the induction of pyrimidine dimers in RA and RA X 10.

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1.16 AMINO ACID SEQUENCES IN THE α CHAINS OF HEMOGLOBINS FROM THREE STRAINS OF MICE

R. A. Popp Diana M. Popp

Introduction. – It has been suggested that the solubility differences of mouse hemoglobins result from amino acid replacements in the α chain. The sequences of amino acids have been determined for the α chains of hemoglobins from three strains of mice. The sequences are reported here.

Methods and Results. – Methods for the separation of the tryptic peptides have been reported previously.[1] The larger peptides were degraded further by chymotrypsin and/or pepsin to produce smaller fragments suitable for Edman degradation. The sequence of amino acids in the α chains of hemoglobins from C57BL, NB, and BALB/c mice are given in Table 1.16.1. Each has 141 amino acids. Simple replacements were found except for position 68 of the BALB/c α chain, which contains a half unit each of Thr and Ser.

Discussion. – The α chain of mouse hemoglobins is homologous to that of the α chains of human and other mammalian hemoglobins studied. There are 16 amino acid replacements when comparing the sequences of the C57BL α chain with that of the human α chain.[2] This is similar to the

Table 1.16.1. Sequence of Amino Acids in α Chains of Mouse Hemoglobins

								αT-1					αT-2					αT-3					
C57BL	–	Val	Leu	Ser	Gly	Glu	Asp	Lys	Ser	Asn	Ileu	Lys	Ala	Ala	Try	Gly	Lys	Ileu	Gly	Gly	His	Ala	
								7				11					16						
											αT-4									αT-5			
	–	Gly	Glu	Try	Gly	Ala	Glu	Ala	Leu	Glu	Arg	Met	Phe	Ala	Ser	Phe	Pro	Thr	Thr	Lys	Thr	Tyr	Phe
											31									40			
NB[a]	–				Val																		
					25																		
														αT-6				αT-7	αT-8				
C57BL	–	Pro	His	Phe	Asp	Val	Ser	His	Gly	Ser	Ala	Gln	Val	Lys	Gly	His	Gly	Lys	Lys	Val	Ala	Asp	Ala
														56				60	61				
NB[a]	–																			Ileu			
																				62			
C57BL	–	Leu	Ala	Asn	Ala	Gly	Ala	His	Leu	Asp	Asp	Leu	Pro	Gly	Ala	Leu	Ser	Ala	Leu	Ser	Asp	Leu	His	Ala
NB[a]	–			Ser																			
				Thr																			
BALB/c[a]	–			Ser																			
				68																			
			αT-9		αT-10							αT-11											
C57BL	–	His	Lys	Leu	Arg	Val	Asp	Pro	Val	Asn	Phe	Lys	Leu	Leu	Ser	His	Cys	Leu	Leu	Val	Thr	Leu	
			90		92							99											
																			αT-12				
	–	Ala	Ser	His	His	Pro	Ala	Asp	Phe	Thr	Pro	Ala	Val	His	Ala	Ser	Leu	Asp	Lys	Phe	Leu	Ala	Ser
																			127				
									αT-13		αT-14												
	–	Val	Ser	Thr	Val	Leu	Thr	Ser	Lys	Tyr	Arg												
									139		141												

[a]Only the positions of amino acid replacements are indicated.

number of replacements also noted for horse[3] or rabbit[4] hemoglobin. The only replacement of special interest is the one at position 68 of BALB/c, which contains half units of Thr and Ser. The genetic significance of this unique situation is under investigation.

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1.17 LINKAGE OF *Es-1* AND *Es-2* IN THE MOUSE

R. A. Popp W. S. McEwen

Introduction. – Petras[1] has described a phenotypic variation of the first prealbumin esterase in serum of a feral mouse. A similar phenotype was not observed in several strains of laboratory mice examined by Petras. The locus that controls the presence or absence of this prealbumin esterase was assigned the symbol *Es-2*. The allele $Es\text{-}2^a$ acts as a null gene, and $Es\text{-}2^b$ permits the synthesis of the prealbumin esterase Es-2b. We wish to report here that strain RFM/Un mice possess the $Es\text{-}2^a$ allele and that *Es-2* is closely linked to *Es-1* in linkage group XVIII.

Methods and Results. – Blood was obtained from the tail or jugular vein. Serum from clotted blood

Table 1.17.1. F_2 and BC_1 Mice Classified for *Es-1* and *Es-2* Genotype

Matings	Offspring: Nonrecombinants				Offspring: Recombinants				Total
	Es-1 a/a	a/b	b/b		a/a	a/b	b/b		
	Es-2 b/b	b/a	a/a		a/b	a/a	b/a		
$\frac{Es\text{-}1^a\ Es\text{-}2^b}{Es\text{-}1^b\ Es\text{-}2^a} \times \frac{Es\text{-}1^a\ Es\text{-}2^b}{Es\text{-}1^b\ Es\text{-}2^a}$	35	67	37		5	5	3	4	156
$\frac{Es\text{-}1^a\ Es\text{-}2^b}{Es\text{-}1^a\ Es\text{-}2^b} \times \frac{Es\text{-}1^a\ Es\text{-}2^b}{Es\text{-}1^b\ Es\text{-}2^a}$	62	55			6		9		132
$\frac{Es\text{-}1^b\ Es\text{-}2^a}{Es\text{-}1^b\ Es\text{-}2^a} \times \frac{Es\text{-}1^a\ Es\text{-}2^b}{Es\text{-}1^b\ Es\text{-}2^a}$		8	11			1		1	21
					Recombination frequency 0.1100 ± 0.0178				

was subjected to starch gel electrophoresis, and the esterases were localized by a diazonium dye-coupling reaction as previously described.[2] Homogenates of several tissues were similarly analyzed. RFM mice ($Es\text{-}1^bEs\text{-}2^a/Es\text{-}1^bEs\text{-}2^a$) were mated to C57BL mice ($Es\text{-}1^aEs\text{-}2^b/Es\text{-}1^aEs\text{-}2^b$), and the F_1 mice were intercrossed or backcrossed to parental strain mice. Serum from the F_2 and BC_1 mice were analyzed to classify them for their Es-1 and Es-2 phenotypes. F_1 mice show an intermediate quantity of prealbumin esterase, which indicates that $Es\text{-}2^a$ and $Es\text{-}2^b$ are codominant alleles. Tissues, such as the liver, kidney, and intestine, which possess large quantities of a similar esterase, also lack Es-2b in mice of $Es\text{-}2^a/Es\text{-}2^a$ genotype. The genotypes of the 156 F_2 and 153 BC_1 individuals examined are shown in Table 1.17.1. The combined recombination frequency of alleles at *Es-1* and *Es-2* was calculated to be 0.1100 ± 0.0178.

Discussion. – Strain RFM/Un mice lack a prealbumin esterase that is present in all other strains of laboratory mice examined to date. The specific function of this esterase is as yet unknown; however, absence of the enzyme does not seem to reduce the viability of such individuals. The analysis establishes that *Es-2* and *Es-1* are linked. Previous studies showed that *Es-1* is in linkage group XVIII;[3] *Es-1* gives 2% recombination with *Os*. The linear order of *Es-1*, *Es-2*, *Hk*, and *Os* remains to be established.

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1.18 HETEROZYGOSITY IN CHROMOSOMES AND IN PUFFS OF *SCIARA OCELLARIS*

Crodowaldo Pavan A. L. P. Perondini

Recently we found an infection in various tissues of *Sciara ocellaris* (see Sect. 1.19). This strain of flies, even without the infection, is of interest because the great majority of individuals which we examined were heterozygous for several loci; this heterozygosity is easily observable in the polytene chromosomes. Some of these heterozygous loci were described by Metz in 1937.[1] We found, however, that in some of these loci one side may be puffed while the other is condensed. We followed the development of one of these puffs (region 7B of chromosome C). It starts at a specific stage of the fourth instar larvae when the eyespots undergo a migration to a lateral position (stage *f*, according to Garcia).[2] Probably, both sides of the chromosomes start together and stay puffed until

stage *h* (when the eyespots are halfway to their final lateral position). After this, one side of the puff condenses rapidly, while the other side stays puffed longer and is more active, as shown by the larger amount of grains in the autoradiograms. At the onset of stage *i* (eyespots in the final lateral position), one side is already condensed and the other is in the process of condensation that is completed during this stage. At present, we are (*a*) studying the frequencies of several of these heterozygous loci in natural populations, (*b*) organizing chromosome maps of the fourth instar larvae, and (*c*) studying the pattern of puffs and DNA synthesis in the salivary gland chromosomes.

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1.19 CHANGES IN CHROMOSOMES INDUCED BY INFECTION IN SCIARA

Crodowaldo Pavan A. L. P. Perondini

An infection by a microsporidian was found in various tissues of a Sciaridae (*Sciara ocellaris*) collected in the greenhouse of our laboratory. In cells of the salivary gland, muscle, fat body, and other tissues, the infection induces a great increase in size of the nucleus and of the chromosomes. In the majority of cases, it induces an increase in polyteny and in puff activity. In the salivary gland, normal puffs do not appear even in slightly infected cells. In the fat body the changes are not only in the size but also in the number of chromosomes: the extra polyteny is followed by polyploidy. In many infected cells the X chromosomes appear to be more active than the autosomes. This situation is similar to that produced in cells of *Rhynchosciara* by another species of microsporidian.[1,2] Infections like this are very frequent in many species of invertebrates, and they should be explored more intensely as we are sure they will permit studies in many fundamental problems of biology.

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1.20 VARIATIONS IN METABOLIC ACTIVITY DURING THE LARVAL DEVELOPMENT OF *RHYNCHOSCIARA*

Ellen M. Mattingly Vivien B. Whitfield

The habit of synchronous development seen in the insect *Rhynchosciara angelae* and related species has been used for the study of metabolic events in relation to larval development and to chromosomal phenomena such as the formation of puffs. DNA synthesis in the salivary gland, as demonstrated by autoradiography, remains extremely vigorous throughout the early development. Other tissues with polytene chromosomes, such as gastric caeca, intestine, and Malpighian tubules, show progressive diminution of DNA synthesis, so that at the stage of formation of the previously described DNA puffs on the salivary gland chromosomes, there is very little evidence of DNA synthesis in the other tissues. The cells that are most actively synthesizing DNA also show the highest degree of incorporation of uridine-^{3}H and leucine-^{3}H.

Particular attention has been given to the labeling of polytene chromosomes with ^{3}H-leucine at different stages of development. The label resulting from incorporation of the radioactive amino acid is associated most strongly with the DNA-positive bands of the chromosomes. After several hours exposure to the isotope, the chromosome puffs also become heavily labeled. The most rapidly labeled nuclear components are the many micronucleoli associated with certain bands of the chromosomes. Fixation with neutral formalin shows that much of the label that is removed by acid fixation is concentrated in these micronucleoli.

1.21 CHROMOSOME ACTIVITY IN THE SALIVARY GLAND OF *RHYNCHOSCIARA* MAINTAINED IN VITRO

L. C. G. Simões Crodowaldo Pavan

Gene activity is represented by two equally important and probably independent events: duplication of DNA and production of specific RNA. The first event is called gene replication and the second gene action. Although there exists considerable information on how these events occur, very little is known about how to regulate or control them. The polytene chromosomes of *Rhynchosciara* are excellent tools to study these problems. F. S. Lara has devised a physiological solution for *Rhynchosciara* in which we can maintain the salivary gland alive in vitro for over one week. Very well-banded chromosomes can be observed in glands maintained in vitro for ten days at room temperature. In this solution we were able to maintain isolated cells which look normal after 5 hr of incubation.

Synthesis of DNA and RNA in cells of whole salivary glands was studied for the first 96 hr in vitro. Although the chromosomes of cells of each region are in the same stage of development, they may show asynchrony in synthesis of DNA. We are trying to synchronize the synthesis of DNA by maintaining the glands in Lara's solution with an excess of thymidine for a certain time. This procedure inhibits DNA synthesis. When the glands are returned to normal medium, DNA synthesis is resumed and many nuclei show differential incorporation of ^{3}H-thymidine. The majority of these nuclei show intense incorporation in heterochromatic regions. This would suggest that in *Rhynchosciara* chromosomes the synthesis of DNA starts independently in different bands, some of which are heterochromatic. These data are, however, preliminary, and experiments are under way to determine the pattern of DNA synthesis in these chromosomes.

1.22 ISOLATION OF POLYTENE NUCLEI

Günter Weirich

A study has been made to find conditions under which dipteran polytene nuclei can be isolated without structural damage. The materials of this investigation were salivary glands of the fly *Rhynchosciara spec.*

After the failure of some large-scale experiments using the procedure indicated previously,[1] an attempt was made to improve the isolation medium. The basic medium for all previous studies has been a rather complex solution developed by Lara and collaborators[2] in approximation of the *Rhynchosciara* hemolymph. It is composed of a total of 38 millimolar amino acids (proline, leucine, valine, alanine, glycine, serine, glutamic acid, aspartic acid), 5 millimolar dicarbonic acids (succinic acid, fumaric acid), 76 millimolar sugars (sucrose, trehalose, glucose), and 46 millimolar inorganic salts, including 10 millimolar $MgSO_4$ and 1 millimolar $CaCl_2$. The pH is usually adjusted to 7.2.

This medium gives very good results in organ culture experiments (see Sect. 1.21), but it is not appropriate to maintain isolated salivary gland nuclei. After isolation by means of micromanipulation in a droplet of this medium, either one or both of two typical denaturation patterns occur. In one case, the chromosomes collapse and stick together in the middle of the nucleus, which itself is slightly shrunk (25 to 30% reduction of the original diameter of 65 to 70 μ). After the alternative denaturation process, the nuclear volume is not changed, while the chromosomes are shrunk to about 60% of their original diameter. They are possibly elongated and extensively attached to the nuclear membrane. The occurrence of these two artifacts is not strictly dependent on osmotic pressure, since they both are found over a wide range of concentrations and usually even in the same preparation.

In a series of experiments, 6 partial mixtures of Lara's medium, 15 different concentrations of the whole mixture, 12 solutions modified in calcium and magnesium content, 8 media enriched with bovine serum albumin or polyvinylpyrrolidone, and 7 other insect tissue culture solutions were tested for their ability to maintain free polytene nuclei. None of them gave better results, but many caused greater damage to the nuclei than the Lara solution. Furthermore, it could be shown that the stability of the nuclei is much more dependent on the physiological stage of the larvae than on differences in the composition of the isolation medium.

It is concluded that there are rather specific factors involved in the structural stabilization of these chromosomes, and an attempt is being made

to obtain data about the composition of the salivary gland cytoplasm.

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1.23 CHANGES IN THE ULTRASTRUCTURE OF SALIVARY GLAND CYTOPLASM IN *SCIARA OCELLARIS* (COMSTOCK, 1882) DUE TO MICROSPORIDIAN INFECTION

Artur Jurand[1] L. C. G. Simões
Crodowaldo Pavan

The effects of infection by a microsporidian, *Thelohania sp.*, on the ultrastructure of the cytoplasm of salivary gland cells in *Sciara ocellaris* were investigated. Uninfected cells in the cytoplasm contain a dense ribosomal population studding large condensations of lamellar and vesicular endoplasmic reticulum, numerous scattered Golgi areas, and typical metazoan mitochondria (Fig. 1.23.1).

In infected cells of the salivary glands the ultrastructural integrity of cytoplasm is profoundly changed due to depletion of ribosomes, disarrangement of endoplasmic reticulum of both lamellar and vesicular types, and decrease in size and increase in numbers of mitochondria. The overall electron opacity of the infected cytoplasm is decreased due to the decrease of ribosomal population and, to some extent, also to formation of cytoplasmic vacuoles, most probably by dilation of endoplasmic reticulum cisternae (Fig. 1.23.2).

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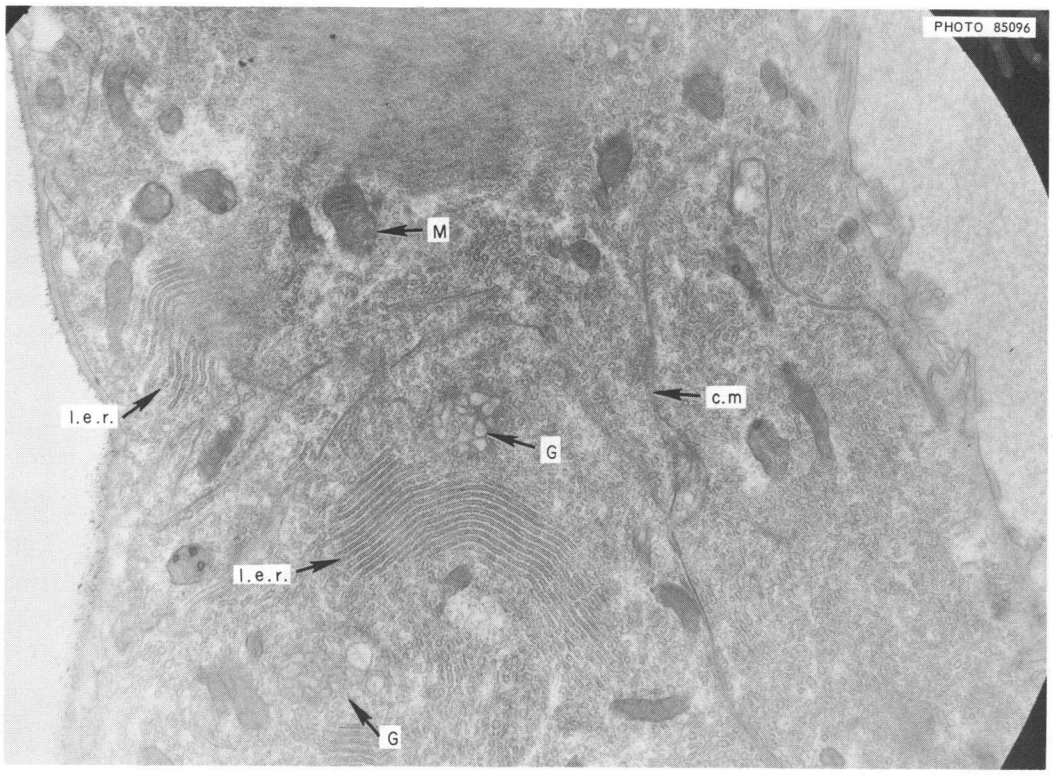

Fig. 1.23.1. Cytoplasm of Normal Salivary Gland Cells. Note large accumulations of the lamellar endoplasmic reticulum cysternae (l.e.r.), Golgi areas (G), motochondria (M), and cell membranes between adjacent cells (c.m.). On the left, basement membrane covering the basal side; on the right, apical cell membrane modified into brush border lining the lumen of the gland. 14,500X.

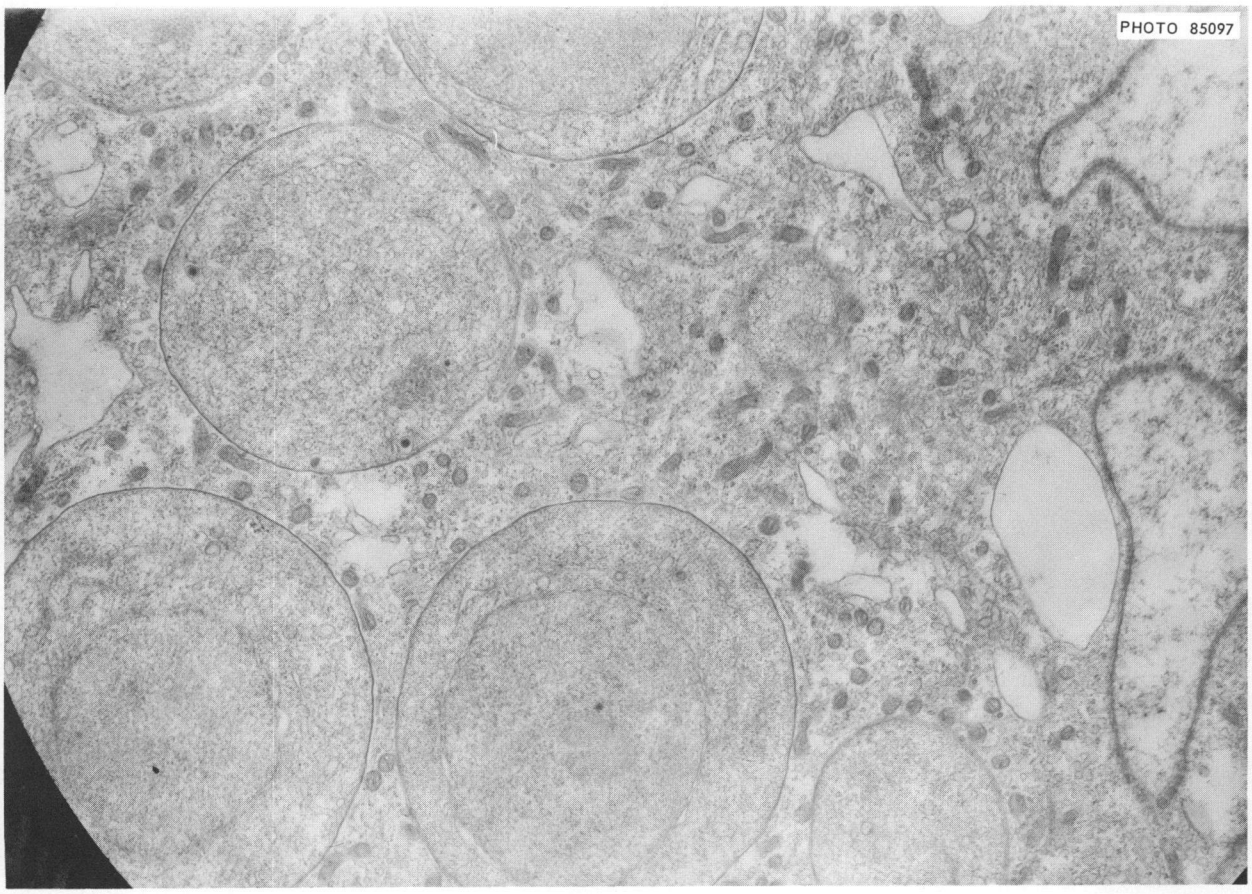

Fig. 1.23.2. Cytoplasm of an Infected Cell. Note large round sections through parasite cells, lower electron opacity of the host cytoplasm, smaller diameter of mitochondria, disorganization of endoplasmic reticulum, and vacuolation of cytoplasm. 14,500X.

Fungal Genetics

F. J. de Serres[a]

Radiation Mutagenesis (2.1)		Chemical Mutagenesis
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2.1 THE REVERTIBILITY OF ULTRAVIOLET-INDUCED *ad-3B* MUTANTS TREATED WITH HYDROXYLAMINE

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

From earlier data[1] it has been possible to demonstrate that uv-induced *ad-3B* mutants fall into three classes: (1) those which revert with nitrous acid and/or ethyl methanesulfonate and which appear to be base-pair substitution mutants, (2) those which revert only or very markedly with acridine mustard ICR-170 and which appear to be the result of base-pair insertions or deletions, and (3) those which fail to revert and cannot be placed in classes 1 or 2 (the majority of these revert spontaneously).

Malling[2] has shown that in *Neurospora*, hydroxylamine induces specifically GC →AT transitions. In the present series of experiments the revertibility of uv-induced *ad-3B* mutants treated with hydroxylamine has been studied in an attempt to to identify the genetic alteration in the base-pair substitution mutants. Drake[3] used hydroxylamine in an extensive test of the revertibility of *r*II mutants induced in bacteriophage T4 by uv and came to the conclusion that the principal uv target in mutagenesis is the GC base pair, since very few of the mutants reverted with hydroxylamine and were thus presumed to have AT at the mutant site.

Results. – (1) None of the mutants previously classified as base-pair additions or deletions (class 2) were found to be revertible with hydroxylamine, (2) only 9 of the 65 base-pair substitution mutants tested reverted with hydroxylamine, (3) two mutants which until now had failed to revert (class 3) did so with hydroxylamine and were thus subsequently included in the class 1 mutants, and (4) in general, rather low reversion rates were found. Only 2/11 mutants tested reverted extremely frequently with hydroxylamine. (The possibility that these two mutants revert by mutation at suppressor loci is being tested.)

Conclusions. – The main value of these results lies in the fact that they make it possible to compare similar data from organisms which differ as markedly as bacteriophage T4 and *Neurospora* in the organization of their DNA. From his work with T4, Drake concluded that approximately 50% of the *r*II mutants induced by uv were the result of base-pair transitions. An estimate of the percentage of base-pair substitutions among *ad-3B* mutants can be made based on the earlier data.[1] At least 39% are base-pair substitutions, and in view of the substantial proportion of mutants which were unclassifiable, the figures agree fairly well with results of Drake.

Only 2/27, or 5%, of the transition mutants studied by Drake were revertible with hydroxylamine in contrast with 11/67, or 16%, of the *ad-3B* mutants. Since the sample of T4 mutants is small, these results seem best interpreted as indicating that there is a similarity in the uv response of T4 and *Neurospora*. If we assume, as with Drake, that the revertants occur at the site of the original mutation, then in *Neurospora* as in T4 most of the uv-induced mutants studied have an AT base pair at the mutant site and uv probably leads to mutation by producing the base-pair transition GC → AT.

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Drosophila Cytology and Genetics

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Radiation Genetics (3.1, 3.2)

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P. A. Roberts

Chromosome Behavior (3.3)

Rhoda F. Grell J. W. Day

Biochemical Genetics and Drosophila Mutants (3.4)

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3.1 A POSITIVE CORRELATION BETWEEN CROSSING OVER IN HETEROZYGOUS PERICENTRIC INVERSIONS AND REDUCED EGG HATCH OF DROSOPHILA FEMALES

P. A. Roberts Mary C. Stewart

In theory, crossing over within heterozygous pericentric inversions produces duplicated-deficient chromosomes; the lowered fertility of female heterozygotes resulting from death of embryos receiving such chromosomes is responsible for the relative infrequency of pericentric inversions in natural populations. Alexander (1952)[1] reported that egg hatch reduction of females heterozygous for a pericentric inversion was less than 10% and suggested that heterozygotes for pericentric inversions may not be at such a selective disadvantage as has been supposed.

We studied recombination and egg hatch of females heterozygous for three different pericentric inversions. A positive correlation between the amount of crossing over within a pericentric inversion and the degree of egg hatch reduction was obtained: Females heterozygous for In(3LR)199 yielded no recombinants within the inversion and had the same egg hatch as controls, while females heterozygous for In(3LR)269 yielded 3% double crossovers within the inversion and showed a 50% reduction in egg hatch; recombination and egg hatch values for In(3LR)165 were intermediate. This suggests that heterozygosity for pericentric inversions can lead to marked reductions in fertility, presumably as a result of the transmission of duplicated-deficient chromosomes to the zygote.

References

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3.2 EVIDENCE ON THE NATURE OF DOSAGE COMPENSATION IN DIPTERA

P. A. Roberts R. F. Kimball[1]
Crodowaldo Pavan[1]

In a preceding report[2] generalized puffing of chromosomes of cells of female larvae of *Rhynchosciara angelae* infected by a microsporidian

was described: the chromosomes of some nuclei appear relatively condensed with dark bands, while in other nuclei the banding pattern has become less distinct as chromosome volume has increased. Increases in size without further increase in DNA content (determined by Feulgen microspectrophotometry) indicate that chromosomes can respond to infection by puffing at many loci. The paired X chromosomes of female larvae, like the autosomes, show some banding throughout most of the stages of enlargement, although distal regions of the X's puff more than proximal.

PHOTO 85099

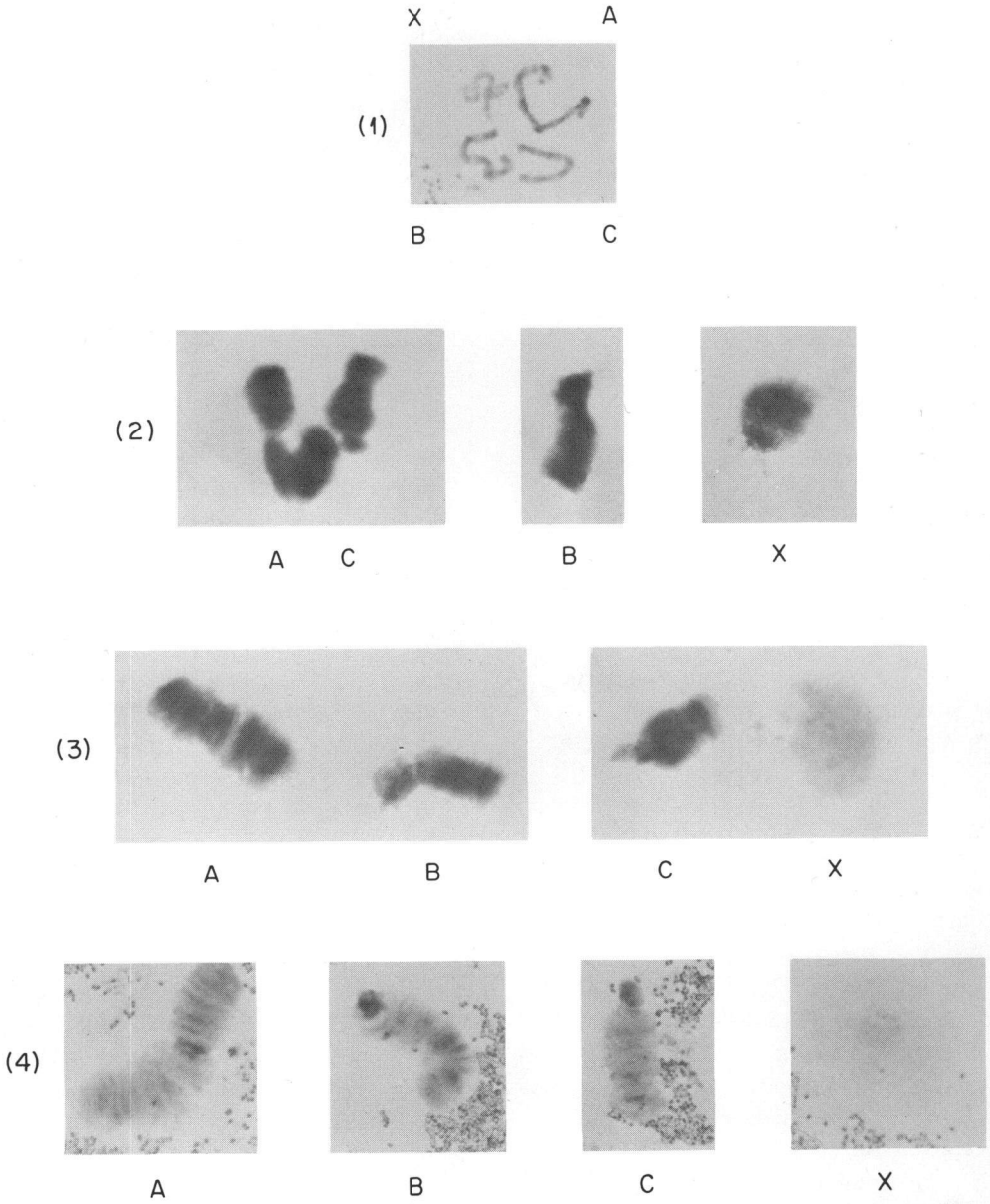

Fig. 3.2.1. Feulgen-Stained Chromosome Complements of an Uninfected (1) and Three Infected Salivary Gland Cells (2–4) of a Male Larva of *R. angelae* Photographed at the Same Magnification. Nucleus 4 has 8 times the DNA of nucleus 1, while nuclei 2 and 3 have 16 times the DNA of nucleus 1.

Studies of chromosomes of infected cells of male Rhynchosciara larvae have been completed. In contrast with the paired X chromosomes of the female, the single X of the male loses its banding early in the process of enlargement and eventually assumes an appearance like that of a faint elliptical nebula which, in some squashed preparations, may exceed the largest autosome in size. The autosomes of Fig. 3.2.1, nucleus 3, are only slightly more puffed than the autosomes of nucleus 2; but the puffing of the X chromosome in nucleus 3 is much more pronounced than in nucleus 2. In nuclei 3 and 4, the X chromosome has lost all traces of its normal banding pattern.

In cells of a species of Sciara infected by a microsporidian, the single polytene X of the male also puffs more than the two polytene X chromosomes of the female. This is further evidence that dosage compensation in Diptera is brought about by increasing the activity of genes throughout the single X chromosome of the male by puffing of the whole chromosome.

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3.3 A CYTOLOGICAL DEMONSTRATION OF NONHOMOLOGOUS PAIRING IN THE OOGONIA OF *DROSOPHILA MELANOGASTER*

Rhoda F. Grell J. W. Day

That nonhomologous chromosomes may associate and segregate from one another in the Drosophila oocyte has now been unequivocally established by genetic means. Since the cytology of the oocyte remains unamenable to analysis, a visual demonstration of this phenomenon is lacking. Recently an investigation of the oogonial stages was undertaken in a search for a cytological manifestation of such pairing events. Our studies have been successful in demonstrating that pairing between nonhomologous chromosomes occurs with high frequencies in the oogonial cell of the proper genetic constitution.

Methods. – Females of five different genotypes, each carrying a single free fourth chromosome and a free X duplication, were utilized. The genotypes were alike except for the particular duplication that was present. The five duplications employed, with their metaphase length (where chromosome 4 is arbitrarily assigned a length of 1), are: 1144 – 1.1, 1337 – 1.4, w^{m5} – 1.9, 856 – 3.0, and z^9 – 3.8.

Ovaries were removed from newly eclosed females, stained with 2% orcein in 45% acetic acid, and squashed. Metaphase figures of oogonial cells were classified as to the presence or absence of pairing between the X duplication and chromosome 4. The nonhomologs were considered to be paired when they were found lying as close to one another as homologs exhibiting somatic pairing normally lie. The type of cyst to which each recorded oogonial cell belonged (i.e., 1, 2, 4, or 8 cell) was also noted. Eight cell cysts at metaphase represent those which upon completion of division will give rise to the oocyte and 15 nurse cells.

Results and Discussion. – Genetic studies[1] with females of similar or identical genotypes to those used in the present studies have shown that frequencies of nonhomologous pairing between the fourth chromosome and the X duplication in the oocyte are extremely high and are positively correlated with the similarity in length of the two nonhomologs. Table 3.3.1 presents the results of the present cytological studies, based upon ~200 figures per genotype. Here we find a similar correlation between pairing frequency and size similarity. If w^{m5} is ignored for the moment, the highest frequency occurs with 1144

Table 3.3.1 Frequencies of X Duplication, Four Pairing in Oogonial Cells

Duplication	Length	Dp, 4 Paired (%)	Number of Cells Analyzed
1144	1.1	45.1 ± 3.5	204
1337	1.4	36.8 ± 3.4	201
w^{m5}	1.9	47.6 ± 3.6	206
856	3.0	26.9 ± 2.8	260
z^9	3.8	27.7 ± 3.2	202

(1.1), 45.1%; the lowest with 856 (3.0), 26.9%, and z^9 (3.8), 27.7%; and an intermediate frequency with 1337 (1.4), 36.8%. Duplication w^{m5} differs from the other four duplications in possessing a fourth chromosome base instead of an X base. Presumably this difference is responsible for its high frequency of association with chromosome 4 (47.6%), which is inconsistent with its size. Interestingly, genetic studies with w^{m5} indicate that the pairing advantage in the gonia, conferred by homology with the 4, does not obtain in the oocyte.

The classification of the cells according to the age of their cyst permitted examination of the possibility of an increasing tendency toward nonhomologous pairing with increasing proximity to the oocyte stage. Nonhomologous pairing in the 4 and 8 cell cysts was 37.7% as compared to 26.4% in the 1 and 2 cell cysts. The difference is significant at the 2% level.

Summary. – Pairing between two nonhomologous chromosomes, one a free X duplication and the other a free fourth chromosome, has been observed cytologically with high frequencies in the oogonial cells. Like distributive pairing in the oocyte, its frequency is correlated with the similarity in size between the two participating chromosomes. Unlike distributive pairing in the oocyte, homology apparently aids in recognition in the gonial cell. An increased tendency toward nonhomologous pairing is observed as the gonia approach the oocyte stage.

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3.4 ELECTROPHORETIC VARIANTS OF α-GLYCEROPHOSPHATE DEHYDROGENASE AND THEIR GENETICS IN *DROSOPHILA MELANOGASTER*

E. H. Grell

The enzyme α-glycerophosphate dehydrogenase (GDH) catalyzes the oxidation of α-glycerophosphate to dihydroxyacetone phosphate and the reverse reaction. Extraordinarily high activity of this enzyme is found in insect flight muscle, and it has been shown to have an important role in the rapid production of energy from carbohydrate by flying insects.

Electrophoretic variants of at least 12 enzymes are known in Drosophila. More than one electrophoretic type for an enzyme is more the rule than the exception in this species. The genetic loci responsible for the variations have in most cases been located on the linkage map of Drosophila.

For this investigation the α-glycerophosphate dehydrogenases were electrophoresed on polyacrylamide gel, and regions of enzyme activity were made visible with a reaction yielding a colored product. It was found that inbred strains of Drosophila may be divided into two types according to the electrophoretic mobility of their GDH. One type contains GDH that migrates more rapidly to the anode than the other. There appears to be a family of enzymes in each inbred. There is a major component and two slower-moving minor components. Larvae, pupae, and adults have the same pattern.

Crosses between the two different types yield hybrids which have at least five components with GDH activity. There are three major components: the two parental ones and a hybrid with intermediate mobility (Fig. 3.4.1). The presence of a

Gdh^R/Gdh^R Gdh^R/Gdh^S Gdh^S/Gdh^S

Fig. 3.4.1. Photograph of Acrylamide-Gel Electrophoresis of α-Glycerophosphate Dehydrogenases of *D. melanogaster*. On the left is the homozygote of *Gdh-Rapid*. On the right is the homozygote of *Gdh-Slow*. In the center are two heterozygotes.

hybrid major component indicates that the GDH molecule is most likely a dimer. The parental major components contain two subunits that are alike; the hybrid contains two unlike subunits.

Genetic analysis shows that the differences in electrophoretic mobility of GDH is based on there being two alleles of a genetic locus. This locus (called Glycerophosphate dehydrogenase, symbol *Gdh*) is located on the second chromosome. On the standard linkage map it is between Sternopleural (map position 22.0) and dumpy (map position 13.0). Homozygotes of the allele Gdh^R have the rapid pattern. Homozygotes of the allele Gdh^S have the slow pattern of GDH. The hybrid pattern is produced by Gdh^R/Gdh^S heterozygotes.

It is likely that the primary structure of α-glycerophosphate dehydrogenase is specified by the *Gdh* locus.

Cytochemistry and Cell Reproduction

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Yeast Genetics (4.1)

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Insect Cytology and Genetics (4.2–4.5)

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Participation in Cooperative Programs: AEC-NASA Space Biology (22.26–22.29)

4.1 SPONTANEOUS MUTATION RATE OF THE tr_{1-1} MUTANT AND ITS SUPPRESSORS

R. C. von Borstel P. D. Panum
Diane J. Goins

Introduction. – The suppressors of the tr_{1-1} mutant belong to the group known as "super-suppressors"; super-suppressors are capable of suppressing any genetic locus but affect only a limited number of the mutant sites in a locus.[1] The tr_{1-1} super-suppressors belong to a unique class that overlaps only rarely with the other classes of super-suppressors.[2]

We have shown that the other classes of super-suppressors have an enhanced mutation rate during meiosis over that of mitosis, while the super-suppressible sites do not.[3] The enhanced mutation rate at meiosis is attributable to mutations of the addition-deletion type,[4] and lack of enhancement is attributable to mutations of the base-substitution type.[5] These facts are consistent with the hypothesis that the super-suppressible mutations are nonsense mutations and the super-suppressor mutations are mutations of loci responsible for transcribing sRNA.[3]

Results. – Reversions of the tr_{1-1} mutants at the locus and the super-suppressor mutations can be readily distinguished, since reversions at the locus have the ability to liberate tryptophan into the surrounding medium, thus feeding tryptophan-requiring cells in the vicinity of the revertant colony. Super-suppressor mutations affecting the tr_{1-1} mutant do not have this capacity.[6]

Revertants from a strain carrying the tr_{1-1} mutation were selected by placing the yeast in a growth-limiting liquid medium in the multicompartmented boxes of de Serres. The growth was limited by lack of tryptophan. Revertants continued to grow as colonies at the bottoms of the compartments. Cells from these colonies were removed with Pasteur pipets and transferred to plates containing synthetic medium minus tryptophan. When revertant colonies had grown they were replica plated to the same type of plates on a background of a tryptophan-requiring strain of yeast. If a colony had feeding ability, a halo of small colonies would arise around the original colony; this colony would be classified as a feeder and a revertant at the tr_1 locus. Nonfeeders were classified as super-suppressors.

Table 4.1.1. Mutation Rates: Reversion from Dependence to Independence

Mutant	Locus of Mutation	Rate	
		Mitosis	Meiosis
		$\times 10^9$	$\times 10^9$
tr_{1-1}	tr_1	25.2	53.8
	Super-suppressors	2.43	41.4

Both the nonfeeders and feeders can be dominant, since they arise by spontaneous mutations in a diploid strain homozygous for tr_{1-1}.

The results of the mutation rate studies during mitosis and meiosis are presented in Table 4.1.1. It can be seen that the super-suppressors of tr_{1-1} exhibit an enhanced spontaneous mutation rate of approximately 17 times during meiosis, whereas the tr_{1-1} mutant itself is merely doubled.

Discussion. – The enhanced reversion rate at meiosis and the dominance of the nonfeeding revertants are not consistent with the hypothesis that these are structural mutants of a gene encoding a protein.[3] There are at least six super-suppressor loci that suppress the tr_{1-1} locus.[2] Therefore, the data are consistent with the hypothesis that the tr_{1-1} super-suppressors transcribe sRNA. It is possible that the nearly complete lack of overlap of the tr_{1-1} super-suppressors with the principal super-suppressor series indicates that the tr_{1-1} super-suppressors are specific for amino acids conferring tertiary structure to the tr_1 gene product. It is equally possible that these two suppressor series represent different nonsense codons.

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4.2 X-RADIATION SENSITIVITY OF *HABROBRACON* OOCYTES AT DIAKINESIS

R. H. Smith Anna R. Whiting

Introduction. – The complex nature of prophase I in meiosis requires that, for greatest significance of irradiation studies, the exact condition of the chromosomes at time of exposure be determined. Previously reported tests[1] of *Habrobracon* oocytes have demonstrated the high resistance of earlier stages in prophase I and the high sensitivity of oocytes in metaphase I, a ratio of 20 to 1. A similar differential radiosensitivity was later reported in other organisms, for example, *Drosophila*.[2,3] The resistant stage in *Habrobracon* and *Drosophila* is probably the diplotene stage. The only report containing evidence contrary to this response is one on Artemia oocytes, where a decrease in sensitivity was shown during the course of meiosis.[4] Cervini and Giavelli found that prophase, mostly diakinesis, is more sensitive than metaphase. We focus our attention here on the sensitivity of diakinesis.

Results and Discussion. – The *Habrobracon* female has four ovarioles, each ending posteriorly in a uterine sac. A well-fed female restrained from ovipositing for 16 hr has in each uterine sac four eggs in the first meiotic metaphase and one in diakinesis. Previous studies have demonstrated that an exposure of 2000 or 2500 r, sufficient to induce dominant lethal changes in over 99% of eggs in metaphase I, has imperceptible effects on oocytes in prediakinesis stages. In the present experiment, hatchability and times of death of embryos were followed closely over six short egg-laying intervals. By cytological examination, the maximum number of metaphase I eggs per female was found to be 16, as expected. At the exposures used (2000 and 2500 r), the number of nonhatching eggs exceeded this maximum number in 59 out of 113 females. The average number of nonhatching eggs exceeding the 16-egg maximum was 19, and the range was up to 23 eggs per female.

Therefore, the diakinesis stage in *Habrobracon* is in the same range of radiation sensitivity as the first meiotic metaphase.

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4.3 TIME OF DEATH OF A TRANSLOCATION SEGREGANT IN *HABROBRACON*

R. L. Amy R. C. von Borstel

Introduction. – Translocations can be induced by x rays in *Habrobracon* males and detected by inherited partial sterility methods. Of the eggs laid by a translocation heterozygote, about 50% will carry a duplication for one chromosome segment and a deletion for another. Under these conditions such eggs would be deficient for some portion of the normal gene complement. An analysis of the developmental history of eggs of this type should make it possible to determine the time during embryogeny when the missing genes act.

Results and Discussion. – Eggs from *Habrobracon juglandis* (T_3 stock) heterozygous for a single translocation were collected and checked for hatchability. Those that failed to hatch were examined under the dissecting microscope (30×) and classified, on the basis of their morphological appearance, according to the system described by von Borstel and Rekemeyer.[1] The results are summarized in Table 4.3.1. Of a total of 1336 eggs collected, 718 did not hatch. The majority (85%) of the latter had reached fairly advanced stages of embryonic differentiation and were classed as death types 3 or 4. Those categorized as 3a and 3b had ceased development at approximately 20 ± 1 hr; those classed as type 4 had reached 23 hr (a) or at least 24 hr (b) of age before dying. It appears, therefore, that this particular translocation involves a part of the genome that exerts its influence during the last one-third of the 29-hr embryonic period.

Table 4.3.1. Embryonic Stages at Which Death Occurred in Nonhatching T_3 *Habrobracon* Translocation Segregants

Stage of Death	Number of Embryos	Percent
1	4	0.5
2	0	
3a	160	22.3
3b	22	3.1
4a	165	23.0
4b	269	37.5
5	2	0.3
Unclassifiable	96	13.3
Total nonhatching (of 1336 laid)	718	

References

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4.4 SPONTANEOUS MUTATION FREQUENCIES IN *HABROBRACON*

R. C. von Borstel Diane J. Goins
R. H. Smith Katherine T. Cain
Anna R. Whiting Ellen S. Sirota
Margaret L. Haye

Introduction. – We have obtained the frequencies of embryo lethals and translocations in the Raleigh wild-type strain of *Habrobracon*.

Methods. – *Raleigh* females were isolated as virgins and tested for the presence of lethals, and females bearing lethals were discarded. Females having high hatchability were mated with *Raleigh* males, and female offspring were isolated as virgins. Large numbers of these *Raleigh* virgins were tested for lowered hatchability. When a female showed lowered hatchability, she was removed for further testing. This testing included four periods of oviposition for determining hatchability, adult survival, and time of death of haploid

embryos. The females were then mated to *lemon* males containing different sex alleles and tested for hatchability, adult survival, sex ratio, and time of death of diploid embryos. If the lowered hatchability were caused by a recessive embryo lethal mutation, then the deaths during embryogenesis would be covered in the diploid. If the lowered hatchability were caused by deletion-duplication segregants from a female heterozygous for a translocation, then deaths during embryogenesis are merely delayed to a later stage of development.[1] If no change in time of death occurred, then these females were classified as semisterile or sterile, depending on hatchability.

Results. – Of the 1392 *Raleigh* virgins tested, 13 were heterozygous for recessive lethal mutations, 4 were heterozygous for translocations, and 4 were semisterile.

Discussion. – These tests for spontaneous mutation and chromosomal translocation frequency are for the entire genome. The recessive lethal frequency for the *Raleigh* strain is 0.0093. The spontaneous mutation frequency was obtained earlier for the *lemon/Raleigh* hybrid.[2] Two obtained from 751 females tested for a frequency of 0.0026; no translocation heterozygotes were found. The difference is significant and suggests that strain differences exist for spontaneous lethal mutation frequency in *Habrobracon*.

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4.5 X-RADIATION INDUCTION OF MUTATIONS IN *HABROBRACON*

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Introduction. – Unfertilized eggs of *Habrobracon* develop into normal haploid males; fertilized eggs develop into diploid females. Techniques have been developed for *Habrobracon* which permit analysis of the entire genome for dominant lethality, recessive lethality, and chromosomal translocations.[1,2] The purpose of this experiment is to determine these parameters for sperm irradiated with 2000 r. Three batches of F_1 virgins were tested at approximately monthly intervals to see if selection operated against mutation heterozygotes held in cold storage.

Results and Discussion. – *Lemon* males were irradiated and crossed with *Raleigh* females pretested for high hatchability. The frequency of dominant lethality with embryo hatchability as a criterion was 0.279, with 6045 eggs being analyzed. The results of the tests for recessive lethal mutations and translocations are shown in Table 4.5.1. It can be seen that the mutation frequencies remained essentially constant in the three replicates. This fortunate circumstance makes it possible to test large numbers of individuals over a period of months, since only a few hundred can be successfully handled and tested at one time.

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Table 4.5.1. Translocations and Recessive Lethal Mutations in Female Progeny from *Lemon* Males X Irradiated with 2000 r

	Replicate 1 (Jan. 31)		Replicate 2 (Feb. 22)		Replicate 3 (Mar. 21)		Total	
	Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency
Translocations	68	0.200	80	0.253	67	0.250	215	0.232
Recessive lethal mutations								
Embryo	25	0.074	37	0.117	26	0.097	88	0.095
Larva	26	0.076	29	0.092	19	0.071	74	0.080
Pupa	13	0.038	13	0.041	13	0.045	38	0.041
Total females tested	340		316		267		923	

Cell Growth and Differentiation

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Control of Cell Differentiation (5.1–5.4)

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Chromosome Ultrastructure

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Vitellogenesis (5.5)

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Biochemistry of Cell Differentiation (5.6–5.11)

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5.1 ELIMINATION OF MITOCHONDRIA DURING WOLFFIAN REGENERATION IN NEWTS

Artur Jurand

It was shown by Stroeva,[1] using light microscope techniques, that in the rat during the lens development mitochondria disappear gradually from cells concurrently with other morphogenetical changes when lens fibers achieve the later stages of differentiation. At these stages mitochondria remain abundant only in the cells of lens epithelium and the equatorial zone.

During the Wolffian lens regeneration, the heavily pigmented dorsal iris marginal cells are transformed metaplastically into lens cells. In the latter part of this process, all the essential subcellular organelles gradually disappear from the transforming cells, including mitochondria, ribosomes, endoplasmic reticulum, and nuclei. In connection with electron microscope investigations of these changes,[2,3] it was noticed that mitochondria undergo elimination by extrusion through the cell membranes into the intercellular spaces. At the time of extrusion they swell, and the outer membranes form multiple membranous structures (Figs. 5.1.1 and 5.1.2).

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Fig. 5.1.1. Mitochondria Locating Close to an Intercellular Space. The cell is in the beginning part of fiber differentiation phase of newt lens regenerate.

Fig. 5.1.2. A Mitochondrion Partially Extruded in the Intercellular Space. The cell is similar to that of Fig. 5.1.1.

5.2 DIFFERENTIAL EFFECTS OF ACTINOMYCIN ON RIBOSOMAL CLUSTERS IN DIFFERENT PHASES OF CELLULAR DIFFERENTIATION OF LENS REGENERATION

Artur Jurand D. H. Reese
Tuneo Yamada

In an earlier work from this group, differential effects of actinomycin D on progress of cell differentiation in different phases of cells involved in lens regeneration were demonstrated.[1] The cells in the multiplication phase, whether they are in the wall of the lens vesicle or in the lens epithelium, are sensitive, but the cells in the terminal cell cycle engaged in fiber differentiation are resistant. One of many possibilities to interpret the differential effects is to assume that the messenger RNA's functioning in the multiplication phase are short lived, while the messenger RNA's functioning in the fiber differentiation phase are long lived. Another possibility, which we

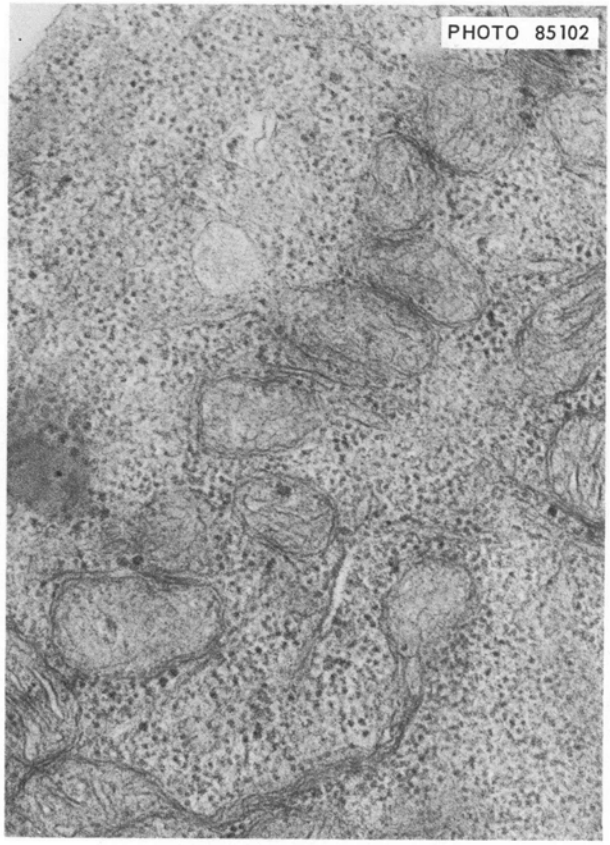

Fig. 5.2.1. A Part of the Cytoplasm of Lens Epithelial Cells Treated with 1 μg/ml of Actinomycin D for 3 hr.

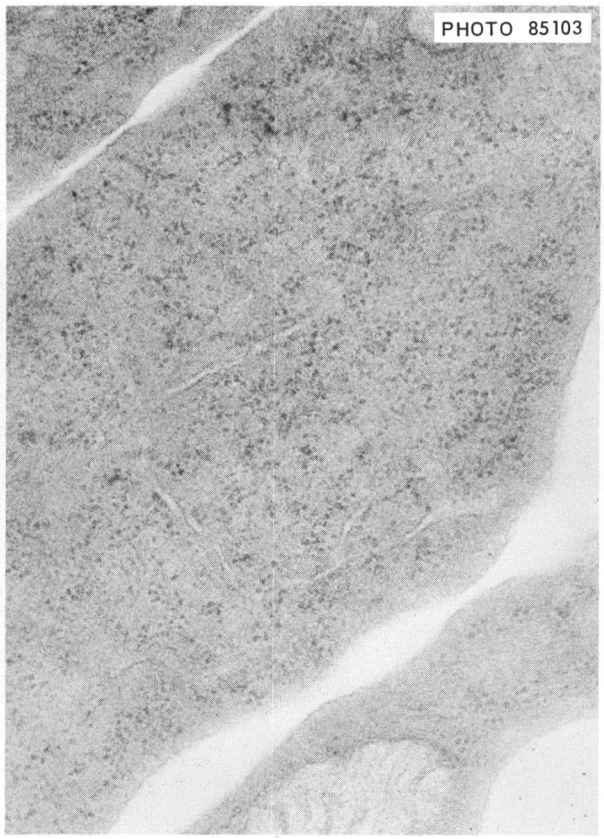

Fig. 5.2.2. A Part of the Cytoplasm of Young Lens Fiber Cells Treated with 1 μg/ml of Actinomycin D for 3 hr.

tended to emphasize, is that actinomycin affects synthesis of ribosomal RNA, and the multiplying cells are more dependent upon production of new ribosomes than the cells in the fiber differentiation phase. As one approach to these problems, we have compared the effect of actinomycin on the pattern of ribosomes as seen in the electron micrographs of thin sections.

Methods and Materials. – The lens regenerates at stages VIII to XII were separated and incubated in vitro for 3 hr at 22°C in Waymouth's MB 752/1 medium containing 1, 5, and 10 μg/ml of actinomycin D. Concurrently, similar regenerates were incubated for the same period of time in the same medium without the antibiotic and served as controls for the experimental series. Further fixation and embedding procedures were the same as in the earlier report.[2]

Results and Discussion. – The 1-μg series indicates that the majority of ribosomes in cells in the multiplication phase are in the monosomal condition, and the large clusters of ribosomes observed in the same cells of the control series[2] are lacking (Fig. 5.2.1). On the other hand, in the young fiber cells of the 1-μg series the medium-sized clusters seen in the control are left intact (Fig. 5.2.2).

In the series of higher concentrations of actinomycin, dense granules of approximately 200-A diameters are frequently seen in the cytoplasm of all cell types (Fig. 5.2.3). Some of them are seen attached to the endoplasmic reticulum. Since they are abundant in the cytoplasmic area, where regular ribosomes are very scarce, one obtains the impression that the large granules are aggregates of ribosomes. In the multiplying cells of the higher concentration series, ribosomal clusters are not obvious, while in the fiber differentiating cells of the same series, some indication of ribosomal clusters is seen.

The data of the 1-μg/ml series, which corresponds to the concentration range used in our first

Fig. 5.2.3. A Part of the Cytoplasm of Lens Epithelial Cells Treated with 10 μg/ml of Actinomycin D for 3 hr.

experiments, indicate that the actinomycin has clear differential effects in inhibiting ribosomal clusters. Since it is probable that the ribosomal clusters are formed in the presence of messenger RNA's, the data suggest a difference in the half-life of messenger RNA's functioning in two phases of cellular alteration. This is in conformity with recent biochemical studies[3,4] which indicate stabilization of messenger RNA's as the lens epithelial cells are transformed into the fiber cells.

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5.3 EFFECT OF "REGENERATING" HUMOR ON LENS REGENERATION

D. H. Reese Tuneo Yamada

A number of investigators have suggested that some substance accumulates in the humor of the lentectomized eye which is responsible for Wolffian lens regeneration. This substance, on the basis of a number of experiments, is assumed to be secreted by the neural retina into the humor of the lentectomized eye, where it comes in contact with the dorsal pupillary margin of the iris. The iris is then "induced" to transform into a lens. Until now no satisfactory demonstration of this substance has been made. The following preliminary experiment was undertaken to determine if this substance could be demonstrated in the humor.

Left lenses were removed from *Triturus viridescens* adults. Seven days after lens removal the humor from each of these eyes was removed with the aid of a fine glass pipet and put into separate capillary tubes (1 mm by *ca.* 1 cm). A piece of normal nonregenerating dorsal iris was put into the humor in each tube. To eliminate evaporation and contact with air, paraffin oil was added to both ends of the tubes so that it was in contact with the humor. The humor with the iris was thus completely enclosed. After 3 days of culture, most of the humor in the tubes was drawn off and replenished with fresh humor from eyes which had been lentectomized 7 days previously. Thus, the pieces of irises were always in humor from 7-day regenerates. Cultures were maintained for 10 to 15 days.

After a few days in culture, the iris pieces had a tendency to round up and were easily fragmented if extreme care was not taken during subculturing. In spite of this, several irises did remain in good condition histologically. Moreover, depigmentation of the dorsal pupillary margin of the iris did occur in one. The degree and extent of depigmentation were consistent with in vivo depigmentation for a comparable period of time. Improvement of the technique is being planned.

5.4 EXPERIMENTS ON LENS INDUCTION IN THE EXTRAEMBRYONIC ECTOBLAST OF CHICK EMBRYOS CULTURED IN VITRO

S. P. Modak Tuneo Yamada

We are reporting here transplantation of early optic vesicles of the chick embryos to an extraembryonic site of the blastoderms cultured in vitro. The work constitutes one of the preliminary experiments for our investigation of the factors involved in lens induction.

Methods. – White Leghorn chick embryos were cultured in vitro by double-ring culture techniques of Gallera and Castro-Correia.[1] Host blastoderms were cultured[2] at primitive streak stages 3^+ and 4. Optic vesicles, with the adjoining lens ectoderm, were dissected out of the donor embryos of 7 to 18 somite stages. Grafts were transplanted to anterior or anterolateral regions in the opaque area, at the same time assuring a close contact between the host ectoblast and the graft. In all, 22 such transplantations were carried out. Three blastoderms were grown for 24 hr, and the rest were sacrificed after 36 to 44 hr development. Blastoderms were fixed in Bouin's fixative, sectioned at 8 μ, and stained in Meyer's hemalum.

Results and Discussion. – A complete breakdown of the results is illustrated in Table 5.4.1. In all cases, graft differentiated well. Graft optic vesicles induced a lens in adjoining lens ectoderm. In all the transplants cultivated for 36 to 44 hr, a lens rudiment, mostly in the form of lens vesicles, was differentiated. In fact, in three cases lenses

Table 5.4.1. Transplantation of the Optic Vesicle and Lens Ectoderm

Stage of Donor Embryo	Number of Grafts	Differentiation of Grafted Material				Reaction of Host Ectoblast		
		Optic Vesicles	Optic Cups	Lens Induction	Induction of Other Sensory Placodes	None	Dorsolateral Ectodermal Type	Induction of Sensory Placodes
7–9 somites	4	1	3	5	2	1	3	2
10–13 somites	7	1	6	7	1	3	4	2
14–18 somites	11	1	10	11	1	5	6	1
Total	22	3	19	23	4	9	13	5

had begun forming primary fibers. Besides lens, at least four positive cases of induction of sensory placodes in the graft ectoderm were observed.

Host ectoblast reacted differently in different grafts. In five blastoderms grafted, optic vesicles induced sensory placodes in the host ectoblast. In these cases a close contact between the graft and the host ectoblast was evident. Thirteen times the host ectoblast showed a typical dorsolateral ectodermal reaction. In these instances the reacting ectoblast was underlined by the packs of mesenchyme. Since our grafts also included some amount of anterior mesenchyme, the latter can be presumed to be responsible for the induction. Only in nine blastoderms was no induction of any kind evident in the host ectoblast, which retained its thin cellular lining. In these cases, the contact between the graft and the host was obstructed by the mesenchyme of graft origin and the extraembryonic mesoblast of the host which moved outward and between the host and the graft.

The next step is to transplant isolated optic vesicles, alone, to the embryonic and extraembryonic ectoblast and, eventually, to grow the whole complex in organ culture on defined media.

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5.5 "PURIFIED" PHOSVITIN FROM VERTEBRATE EGGS

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In order to study the hepatic synthesis and the selective follicular transport of phosvitin into the developing vertebrate oocyte, we have developed as a first step a general method for the isolation and purification of this highly phosphorylated egg-yolk protein.[2] As a second step, we would like to prepare fluorescent and ferritin-labeled antibodies against "purified" phosvitin.

The purification procedure consists of (1) isolation of a phosvitin-lipovitellin complex, (2) ammonium sulfate precipitation of the lipovitellin, and (3) DEAE-cellulose chromatography of the remaining "crude" phosvitin. Chromatograms of "crude" phosvitin from the eggs of five representative vertebrates are provided in Fig. 5.5.1. "Purified" phosvitins were recovered from those portions of the effluent indicated by arrows and were subjected to sedimentation studies and chemical analyses (Table 5.5.1). In all cases the "purified" product displayed a single symmetrical peak in the ultracentrifuge and, by assuming the same diffusion coefficient and partial specific volume as found for chicken phosvitin,[3] all products were found to have a molecular weight of about 40,000 except the trout phosvitin (24,000). The chemical analyses (Table 5.5.1) demonstrate that the "purified" preparations have the highest phosphorus

Table 5.5.1. Sedimentation Coefficients and Chemical Analyses of "Purified" Phosvitins

Animal Source	"Purified" Phosvitin				Fraction *a*, Nitrogen/ Phosphorus	Fraction *b*, Nitrogen/ Phosphorus
	$s^{o}_{20,w}$	Percent Nitrogen	Percent Phosphorus	Nitrogen/ Phosphorus		
Ichthyomyzon unicuspis (lamprey)	3.21	12.05	9.28	2.86		
Salmo gairdneri (trout)	2.28	11.92	9.55	2.79		
Rana pipiens (frog)	3.30	15.56	9.32	3.68	3.73	2.84
Chelydra serpentina (turtle)	3.29	13.67	8.69	3.48	3.55	2.65
Gallus gallus (chicken)	3.20	12.14	11.01	2.44	2.46	2.40

content and lowest atomic nitrogen/phosphorus ratio reported to date for similar species.

The chromatographic patterns presented in Fig. 5.5.1, however, indicate that not in all cases do the phosphoprotein-phosphorus values parallel the absorbancy traces at 280 mμ in the phosvitin region (e.g., Fig. 5.5.1*a*, *c*, *d*). Also, for the frog (Fig. 5.5.1*c*) and turtle (Fig. 5.5.1*d*), at least two well-defined phosphorus-containing components are indicated in the phosvitin fraction. In order to examine this further, "purified" phosvitins from the frog, turtle, and chicken were each subdivided into two fractions, as indicated by *a* and *b* on the chromatograms (Fig. 5.5.1*c*–*e*), and nitrogen/phosphorus ratios for the fractions were determined. The data demonstrate (Table 5.5.1) that in all cases, fraction *b* is more phosphorylated than *a* and that a heterogeneity exists in the "purified" phosvitin. The least difference between the nitrogen/phosphorus ratios for fractions *a* and *b* was found for the chicken, and this difference may well be within the experimental error for the determinations. Nevertheless, fractions *a* and *b* of the chicken phosvitin rechromatographed in their original positions (Fig. 5.5.1*f*).

Thus, phosvitin preparations, in the purest form achieved to date, still seem to be heterogeneous. Whether this heterogeneity is primarily to be found in the protein moiety or, rather, in the extent of phosphorylation is still open to further investigation. This is a factor which must be defined before reliable immunological work can be undertaken. Such future investigations are particularly necessary since our present "purified" phosvitin preparations are relatively nonantigenic and large amounts of material must be administered to elicit an antibody response, thus increasing the possibility of an immunological response to minor contaminants and ill-defined subfractions.

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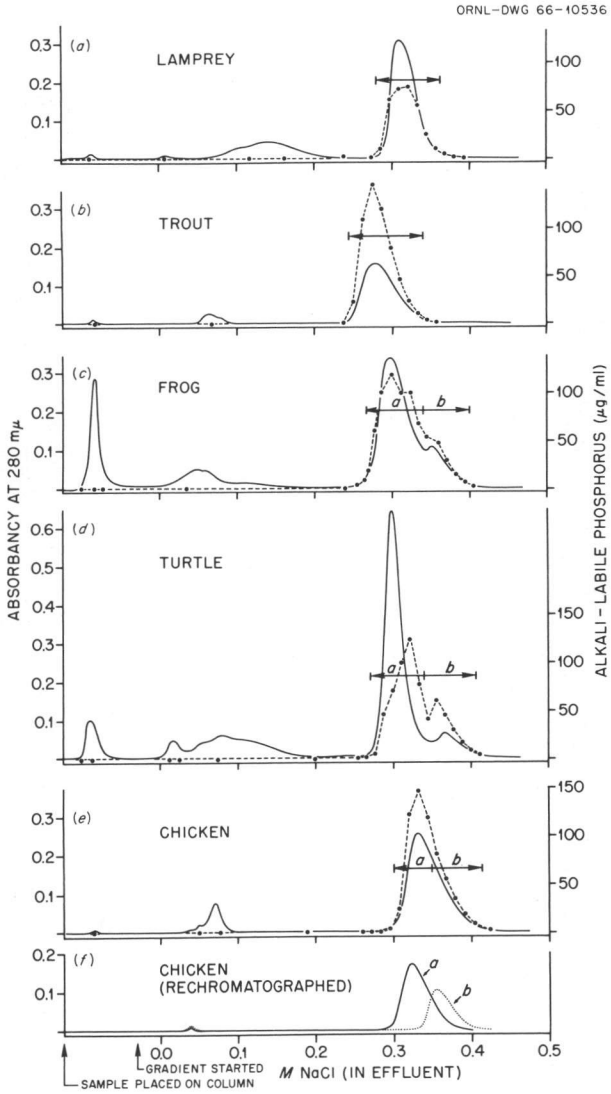

Fig. 5.5.1. (*a*–*e*) **Chromatograms of "Crude" Phosvitin from Five Representative Vertebrates on DEAE-Cellulose.** The solid lines indicate the absorbancy of the effluent at 280 mμ, and the dashed lines indicate values for phosphoprotein-phosphorus determinations. (*f*) Rechromatography of fractions of "purified" chicken phosvitin.

5.6 DIFFERENTIATION OF LENS CELLS IN TISSUE CULTURE – FIBROGENESIS

Dorothy M. Schenck John Papaconstantinou

Introduction. – The vertebrate lens is an evascular tissue composed of a single outer layer of epithelial cells which differentiate into elongated fiber cells. During the differentiation of the lens epithelial cells to lens fiber cells, there occur both morphological and biochemical changes. Thus, morphologically the cell elongates; there is an increase in ribosomal population,[1,2] the cell nuclei and nucleoli enlarge,[3] and there is a loss of mitochondria.[4,5] After fiber cell formation the nucleoli and nucleus decrease in size.[3] All these observations suggest the occurrence of significant changes in protein and nucleic acids during fiber cell formation. Both protein and nucleic acid changes have been reported.[6,7] With respect to protein synthesis, it has been shown, for example, that γ-crystallin synthesis is specifically associated with fiber cell formation.[6,7] In addition, it has been shown that fiber cell formation leads to the suppression of LDH-5 synthesis and results in the persistence of LDH-1.[8] Thus, with respect to protein synthesis associated with fiber cell formation, there are two well-established examples of regulation, that is, the initiation of γ-crystallin synthesis and the suppression of LDH-5 synthesis.

Similarly, there are now well-established examples of alterations in nucleic acid synthesis during fiber cell formation. It has been shown, for example, that m-RNA synthesis in the epithelial cells is potently inhibited by actinomycin D, whereas this is not the case in the fiber cell.[9] These observations have been interpreted to indicate that fiber cell formation is associated with the stabilization of m-RNA. Furthermore, it has been shown that simultaneously with the stabilization of m-RNA there occurs a breakdown of ribosomal RNA.[10]

These protein and nucleic acid alterations associated with lens fiber cell differentiation are excellent biochemical markers for distinguishing between epithelial cells and fiber cells. In view of this, experiments have been conducted to establish the lens epithelial cells in tissue culture and to define the conditions under which the epithelial cells will differentiate to fiber cells in vitro. Furthermore, it is the purpose of this research program to show through biochemical analyses that the morphological changes observed in culture are actually fiber cell formation.

Results and Discussion. – Attempts have been made to induce calf and adult lens epithelial cells to undergo fiber cell differentiation in tissue culture. In view of the recent report of Konigsberg and Hauschka[11] that collagen is an important factor in conditioned medium and that muscle cells

will undergo rapid cellular differentiation when culture vessels are coated with collagen, similar experiments were carried out with the calf lens epithelial cells. Although coating the culture dishes with collagens did not stimulate the formation of fiber cells, it proved to be extremely useful as a factor for the support of rapid growth and proliferation of the lens epithelial cells. Without a collagen layer it took as long as 48 hr for the cells to begin to migrate and proliferate; in the collagen-treated vessels the epithelial cells always proliferated within 24 hr.

Other attempts have been made to induce fiber cell formation in tissue culture. Philpott and Coulombre[12] have reported that cellular elongation of chick lens epithelial cell explants is enhanced by a neural retinal factor. In addition, elongation was also enhanced by placing serum in the culture medium. In experiments done in our laboratory we have supplemented our tissue culture media with both vitreous and aqueous humor extracted from the calf eye. (The tissue culture medium used is Hanks' salts with Eagle's minimal medium.) It was found that upon adding vitreous humor to the tissue culture medium, the calf epithelial cells showed a tendency to orient and elongate. On the other hand, upon the addition of aqueous humor to the culture medium, there was a very limited cellular elongation. The specificity of the effect of vitreous humor on these cells was further shown by treating RA epithelial cells derived from human amnion tissue with vitreous. The vitreous appeared to be slightly toxic to these cells, and in no case was there any observable cellular elongation.

We have concluded from these preliminary experiments that vitreous humor contains some factor or factors which support lens epithelial cell elongation in culture. This factor is not found in aqueous humor. Whether these cellular elongations actually represent fiber cell formation has not been proved. Experiments are now in progress to determine whether these cells show an initiation of γ-crystallin synthesis, suppression of LDH-5 synthesis, stabilization of m-RNA, and ribosomal breakdown. These biochemical characteristics, if positive for the elongated cells, would prove that fiber cell differentiation has indeed occurred in vitro. It is our purpose in carrying out these experiments to regulate fiber cell differentiation so that we may study the mechanisms which determine this morphological change.

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5.7 AN IMMUNOFLUORESCENT ANALYSIS OF LENS CELL DIFFERENTIATION IN VITRO

R. E. Shore John Papaconstantinou

Through the work of Schenck and Papaconstantinou, conditions have been established in which calf and adult lens epithelial cells can be maintained in tissue culture over a period of several years (35 passages). In addition, conditions have been worked out in which both primary cultures and cells established in culture can be induced to form cellular elongations. Morphologically, these elongated cells may represent the differentiation of epithelial cells to fiber cells. It is our purpose to study this phenomenon of cellular elongation in tissue culture, on the molecular level, to determine whether they are characteristic of this stage of differentiation in vivo.

It has been established that γ-crystallin synthesis is associated with fiber cell differentiation. In preparation for our studies on lens epithelial cell elongation in tissue culture, Shore has prepared fluorescent antibodies to purified γ-crystallins, as well as to the α- and β-crystallins.

Preparation of Pure α-, β-, and γ-Crystallins. – Homogenates from calf lens fiber cells were fractionated into α-, β-, and γ-crystallins by a stepwise elution system on DEAE-cellulose. The individual fractions were precipitated by ammonium sulfate, and each fraction was subjected to further purification before being used for antibody production.

γ-Crystallins: After precipitation in ammonium sulfate, the γ-crystallins were further purified by passing them through a bio-gel column. In this step any contaminating α- and β-crystallins could be eliminated. The γ-crystallins obtained by this treatment were tested for purity by electrophoresis on cellulose acetate strips. Under these conditions only one sharp protein band was demonstrated. This fraction was used to prepare antibodies. The specificity of the γ-crystallin antibody produced was tested by the Ouchterlony agar diffusion technique. It was found that no precipitation bands were formed against α- or β-crystallin antigens. A very strong, single precipitation band was obtained when the same sample used to prepare the antibody was tested. These data indicated that a highly specific antibody to γ-crystallin had been obtained.

The anti-γ-crystallin serum was conjugated by the usual procedure with fluorescent reagents, and the conjugated protein was treated by gel filtration. This procedure was carried out to remove the unreacted dye. After removal of the unconjugated dye, the conjugated antiserum was again tested for its specificity. Preliminary experiments have shown that the conjugated antiserum retained its specificity for γ-crystallin. In addition, preliminary experiments have been carried out using preparations of fiber cells and "nonelongated" epithelial cells in tissue culture. These experiments have shown an intense fluorescence in the fiber cells; no fluorescence could be detected in the cultured epithelial cells. Experiments are now in progress to determine whether cellular elongation in tissue culture results in the initiation of synthesis of γ-crystallins. In addition, antiserum to the α- and β-crystallins are being prepared to facilitate studies on the regulation of synthesis of these proteins in cells in tissue culture. It is the purpose of these studies to determine (1) whether cells in continuous culture continue to synthesize α- and β-crystallins; (2) whether α- and β-crystallin synthesis is progressively repressed and how long it takes for complete repression; (3) the kinetics of protein synthesis under conditions of induction and repression.

5.8 FORMATION OF STABLE RNA TEMPLATES IN LENS CELL DIFFERENTIATION

J. A. Stewart John Papaconstantinou

Introduction. – The differentiation of lens epithelial cells to fiber cells represents a terminal stage of cellular differentiation. The epithelial cell is characterized by its ability to replicate and by its basophilic endoplasmic reticulum.[1] It contains metabolically active nucleus and nucleoli,[2] and there is a significant turnover of DNA[3] and RNA.[3] Just prior to the initiation of fiber cell differentiation the epithelial cell goes through one replication cycle[4] and then begins to elongate. This elongation represents the beginning of terminal cellular differentiation. After fiber cell formation, the nucleus disintegrates and the ribosomal population decreases. In view of the fact that the fiber cell represents a nonreplicative cell, experiments were carried out to determine whether there is any significant difference between the stability of the RNA templates in the epithelial cell and the fiber cell. A preliminary report of this work has appeared.[5]

Results and Discussion. – A series of studies were carried out to determine whether the synthesis of lens proteins (α-, β-, and γ-crystallins) in the epithelial cells and fiber cells exhibits a differential sensitivity to actinomycin D. These experiments were designed to show whether the stability of RNA templates in the lens epithelial cell increases when this cell has differentiated to a fiber cell. Intact lenses were incubated in the presence of actinomycin D (10 μg/ml) and ^{14}C-amino acid algal protein hydrolysate. The epithelial cells were separated from the fiber cells after a 2-hr incubation period, and the α-, β-, and γ-crystallins from these two cell types were fractionated on DEAE-cellulose columns. Each fraction was also counted for radioactive protein. The specific activity of the individual crystallins from actinomycin-treated and control lenses was calculated. These data are shown in Table 5.8.1.

Through this type of analysis it was found that the synthesis of the lens proteins in the epithelial cells is potently inhibited, whereas the synthesis

Table 5.8.1. Effect of Actinomycin D on Lens Protein Synthesis

Lens Protein	Epithelial Cells (counts/min per mg of protein)			Fiber Cells (counts/min per mg of protein)		
	Control	Actinomycin (10 μg/ml)	Inhibition (%)	Control	Actinomycin (10 μg/ml)	Stimulation (%)
α-Crystallins	1590	315	80	340	690	103
β-Crystallins	962	166	83	99	165	66
γ-Crystallins	1980	572	71	235	396	68

of these same proteins in the fiber cells is stimulated. These observations indicate that the RNA templates of lens epithelial cells are actively turning over, whereas in the fiber cell these same templates become stabilized.

It can be seen from the data in Table 5.8.1 that the actinomycin caused a stimulation of lens protein synthesis in the fiber cells. In view of the role of lens epithelial cells in the transport of metabolites and nutrients, it is possible that actinomycin may exert some effect on the regulation of this transport in addition to its role as an inhibitor of RNA synthesis. To determine whether this is the case, the experiments reported above were repeated using lenses from which the epithelial cells were carefully removed. The effect of antinomycin on crystallin synthesis in the fiber cells was determined. It can be seen from the data in Table 5.8.2 that upon removal of the epithelial cells there is neither inhibition nor stimulation of crystallin synthesis in the fiber cell. It has been concluded from these experiments that the differentiation of the lens epithelial cell to a fiber cell involves the stabilization of the RNA templates.

Although the data indicate that protein synthesis in fiber cells occurs on stable templates, experiments were carried out to determine whether there is any RNA turnover under the conditions in which actinomycin D exerts its stimulatory effect. Lenses were incubated in the presence of ^{14}C-uridine with and without actinomycin D (10 μg/ml). The epithelial cells and fiber cells were phenol extracted, and the RNA was further resolved by sucrose density gradient centrifugation. Each fraction was counted for radioactivity, and it was found that in the control cells there is a very active RNA turnover in the epithelial, whereas

Table 5.8.2. Effect of Actinomycin D on Lens Proteins Synthesis in Calf Cortex Fiber Cells After Removal of Epithelial Cells

Experiment	Control (counts/min per mg of protein)	+Actinomycin D (counts/min per mg of protein)	Inhibition (–) or Stimulation (+) (%)
1	29	34	+11.7
2	50	37	–16.0

the turnover in fiber cells is significantly decreased. In actinomycin-treated cells the RNA turnover in epithelial cells was inhibited by more than 95%. In the fiber cells there was essentially a 99% inhibition of RNA turnover. Thus, under conditions in which actinomycin D stimulates protein synthesis in the fiber cell, there is virtually no RNA turnover. It has been concluded from these experiments therefore that (1) lens fiber cells contain stable RNA templates, and epithelial cell templates are relatively short lived; and (2) the differentiation of an epithelial to a fiber cell triggers the stabilization of m-RNA.

Experiments are in progress to determine the mechanism of this stabilization.

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5.9 AN ANALYSIS OF THE RNA IN EMBRYONIC AND ADULT CHICK LENS

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Studies on the RNA's of the calf lens epithelial cell and fiber cell have shown that there are significant quantitative differences between the s-RNA and R-RNA of these cells. By the use of MAK columns it was found that the s-RNA makes up approximately 10% of the total phenol-extracted nucleic acids (RNA and DNA), whereas in the fiber cells the amount of RNA in this fraction increases to 60% of the total phenol-extracted nucleic acids. Simultaneously, with the rise in s-RNA there is a sharp drop in R-RNA. These observations have been interpreted to indicate that there is a sharp decrease in ribosomes in the lens fiber cell and that this increase in s-RNA is due to the accumulation of a ribosomal breakdown product having the same MAK properties as t-RNA.

One of the most interesting differences between the lens epithelial cell and the fiber cell is the decrease in nuclear activity when this differentiation occurs. Thus, when nuclear activity stops in the fiber cell, protein synthesis continues at a much slower rate on stable RNA templates. It is also at this stage of differentiation that the decrease in ribosomes occurs. This may be due to the cessation of R-RNA synthesis, but not its degradation. However, it is interesting that during this breakdown of the ribosomes, a significant amount of protein synthesis continues. Thus, the synthesis of proteins in the fiber cells, which occurs on stable templates, must occur on a completely stabilized protein-synthesizing complex. Since m-RNA and R-RNA synthesis are turned off, it is highly probable that t-RNA synthesis is also turned off. The protein synthesis that does occur under these conditions must occur, therefore, not only on a stable template, but on a stabilized protein-synthesizing complex of R-RNA, m-RNA, and t-RNA.

Since the differences in s-RNA and R-RNA occur between cells which have active nuclei as opposed to cells which have inactive nuclei, that is, between epithelial cells and fiber cells, the question arises as to whether metabolically active fiber cells would show nucleic acid characteristics similar to those of the epithelial cells. An answer to this question would further substantiate the association of stabilization of m-RNA and ribosomal breakdown with nuclear inactivation. One way to answer this question would be to analyze the lenses (fiber cells) of early embryos, where the fiber cells are younger and are more active metabolically. Studies on embryonic chick lenses have shown the nuclei of the cortex fiber cells to be metabolically active.[1]

In view of this it was decided to analyze the lenses of chick embryos at various stages of embryonic growth as well as the lenses of post-natal chicks and adults. Because of the smallness in size of the early chick lenses, epithelial cells and fiber cells were not separated. However, the ratio of epithelial cells to fiber cells is so small, when analyzing the nucleic acids by column chromatography, the contributions made by the epithelial cells are not significant.

In a survey study using the MAK profiles of chick lenses at various ages, it was found that there is very little ribosomal breakdown in the embryonic lenses (14 day). Thus the fiber cells, when metabolically active, do not show the same properties as those which have become stabilized, and it appears as if the stabilization of protein synthesis and the breakdown of R-RNA is a property determined by nuclear activity. Furthermore, the survey has shown that as the lenses increase in size (age), the pattern shows an increasing trend toward the adult fiber cell type. When epithelial cells alone, from the adult lenses, were studied, there was no evidence of ribosomal breakdown. From these observations it was concluded that ribosomal breakdown may be limited to the fiber cells in which nuclear metabolic activity has been reduced or turned off completely.

Experiments have been initiated to study the mechanism of ribosomal breakdown in the fiber cells. One approach to this study is to treat R-RNA with RNase under strictly controlled conditions of temperature and time of digestion, to determine whether a breakdown product similar to that found in fiber cells could be produced. These procedures have been used in similar studies with reticulocyte[2] and yeast[3] ribosomal RNA. These procedures are presently being worked out in this laboratory with chicken liver R-RNA and will then be extended to lens R-RNA. The products of controlled RNase treatment of chicken liver R-RNA have been fractionated on MAK columns. The data from these experiments thus far indicate that there are discrete steps in the digestion of R-RNA with RNase and that products similar in chromatographic properties to that of

the ribosomal RNA breakdown product of the lens fiber cell are detected. Further experiments are being done to perfect this technique before applying it to lens R-RNA.

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5.10 RELATIONSHIP BETWEEN CARBOHYDRATE METABOLISM AND PROTEIN SYNTHESIS IN CALF LENS CELLS

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The formation of lens fiber cells is associated with such macromolecular changes as the initiation of γ-crystallin synthesis,[1,2] the stabilization of m-RNA,[3,4] and the breakdown of the ribosomes. Through cytological electron microscopic[5,6] and biochemical studies,[7,8] it has been shown that fiber cell formation also results in the loss of mitochondria. Thus, glycolysis becomes the major pathway of carbohydrate metabolism and the major source of ATP in the fiber cell. On the other hand, a major part of ATP synthesized in the epithelial cells is through oxidative phosphorylation. In view of these major differences in metabolic pathway between the epithelial cells and fiber cells, experiments were designed to determine the respective roles of glycolysis and respiration in the synthesis of lens proteins. To achieve this, the synthesis of α-, β-, and γ-crystallins was studied either under anaerobic conditions or in the presence of oxamic acid, the latter being a specific inhibitor of glycolysis.

Synthesis of α-, β-, and γ-Crystallins Under Anaerobic Conditions. – In these studies lenses were incubated under aerobic and anaerobic conditions in the presence of ^{14}C-amino acids from an algal protein hydrolysate. After the incubation the crystallins from the epithelial cells and fiber cells were fractionated and counted for their radioactivity. The α- and β-crystallins of the epithelial cells were inhibited to the same extent, by about 65%. Thus, approximately 35% of the rate of synthesis of these proteins may be supported by glycolysis. A similar degree of inhibition (60%) of the α- and β-crystallin synthesis was observed in the fiber cells. It appears, therefore, that even though the fiber cells cannot carry out oxidative phosphorylation, glycolysis alone is not sufficient to support the rate of synthesis of these structural proteins observed under aerobic conditions. These observations indicate that α- and β-crystallin synthesis may be regulated by the overlying epithelial cells, possibly through the transfer of ATP.

The γ-crystallins, on the other hand, whose synthesis is initiated during fiber cell differentiation, reacted differently to anaerobic conditions. The γ-crystallins detected in epithelial cell homogenates are synthesized by cells which have started to differentiate into fiber cells. Under anaerobic conditions these proteins proved to be slightly less inhibited than the α- and β-crystallin in that they were inhibited by 52%. In the fiber cells, however, γ-crystallin synthesis was only inhibited by 26%. Thus, the structural proteins which characterize the initiation of fiber cell differentiation, or the formation of a predominantly glycolyzing cell, show a progressive loss of dependence on oxidative phosphorylation. These observations indicate that there may be a regulatory mechanism involving the synthesis of lens proteins and determined by the predominating pathway of carbohydrate metabolism.

Synthesis of α-, β-, and γ-Crystallins During the Inhibition of Glycolysis. – To further substantiate the data observed for protein synthesis under anaerobic conditions, the synthesis of the crystallins was studied under conditions in which glycolysis was specifically inhibited, that is, without affecting respiration. Glycolysis was specifically inhibited by the use of sodium oxamate. Using the same incubation and fractionation conditions as described above, preliminary experiments have shown that the epithelial cell α-crystallins were inhibited by 57% and the β-crystallins by 75%. Since the inhibition of these proteins was higher than expected for the epithelial cells, experiments are in progress to determine whether the concentration of inhibitor (0.08 *M*) used was too high.

The data for γ-crystallin synthesis, on the other hand, again proved to be interesting. Oxamate (0.08 *M*) inhibited the synthesis of these proteins (in elongating cells) by 81%. These observations show that the γ-crystallins are most susceptible to the inhibition of glycolysis.

Analysis of crystallin synthesis in the fiber cells during oxamate treatment showed that all

three groups of proteins were inhibited by 70 to 75%. Since glycolysis is the major source of ATP synthesis in the fiber cells, it would be expected that all protein synthesis be equally inhibited by a glycolytic inhibitor. This is essentially what these experiments showed.

It has been concluded from these preliminary experiments that γ-crystallin synthesis may be regulated by the degree of glycolysis in lens cells. Furthermore, since the synthesis of these proteins occurs during the initiation of fiber cell formation, which is essentially the formation of a predominantly glycolyzing cell, it is possible that an intracellular compartmentalization of ATP is involved in the regulation of synthesis of these tissue-specific lens proteins. Further experiments are being carried out to substantiate the data reported above as well as to determine the factors involved in the regulation of γ-crystallin synthesis.

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5.11 PHYSICAL AND CHEMICAL PROPERTIES OF α-CRYSTALLINS

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The α-crystallins are a group of structural proteins synthesized in the vertebrate lens. In the bovine lens these proteins have a molecular weight of 1×10^6 and are composed of subunits having a molecular weight of 25,000.[1-5] The α-crystallins can be separated from the other crystallins by isoelectric precipitation[1] at pH 5 or by column chromatography using either DEAE-cellulose[2,6] or Sephadex. In the course of our studies on the isolation of these proteins, it was observed that when eluted from a DEAE-cellulose column these proteins are associated with a small amount of RNA. Experiments have been initiated to determine whether this RNA is specifically bound to the α-crystallins and to characterize the RNA by physical and chemical methods.

In order to avoid any chemical treatment that may strip the RNA from the protein, attempts were made to isolate the α-crystallins without varying the ionic strength of the homogenates. Lenses were homogenized in either 0.01 *M* Tris or 0.005 *M* phosphate buffer pH 7, and the homogenates were cleared by centrifugation at 105,000 *g* for 2 hr. The clear supernatant was placed on a Sephadex G-100 column, and the first fraction eluted contained the α-crystallins. Ultracentrifugation of this fraction showed that there was some β-crystallin present; the α-crystallins had an $S_{20,w}$ of 18.8 and the β-crystallin an $S_{20,w}$ of 8.8. An orcinol test on this fraction (5 mg of protein) revealed that there is 6 μg of RNA per milligram of protein. Approximately 400 mg of this fraction was phenol extracted, and an RNA fraction was obtained. This RNA was found to have an $S_{20,w}$ of 3.5. In view of the fact that some β-crystallins were present in this fraction, it is still not certain that this RNA is associated with the α-crystallins. Experiments are in progress to determine whether there is an RNA-protein association and to determine the chemical and physical characteristics of this RNA.

Physicochemical Properties. – It has been reported from this laboratory that isoelectrically precipitated α-crystallin can be resolved into five peaks on DEAE-cellulose.[2] These fractions all gave strong reactions with antibodies to the isoelectrically precipitated fraction. It has not been shown, however, whether the five α-crystallins obtained by DEAE-cellulose fractionation are chemically or immunochemically distinct. Because of the complexity of this macromolecule it is possible that the five peaks observed on DEAE fractionation may be due to changes in the tertiary structure. Alternatively, this diversity may be explained by the amount of RNA bound to the molecule.

Immunochemical Properties. – Detailed analyses of the antigenic properties of the α-crystallins have been carried out as a part of our studies on the physicochemical properties of this molecule.

It has been shown in this laboratory and others that α-crystallins may be dissociated into their subunits by treatment with sodium dodecyl sulfate (SDS) or 7 *M* urea. When these subunits, produced by SDS treatment, were tested antigenically, it was found that they still react with the antibodies to the intact α-crystallin. However, by agar diffusion techniques it was found that this reaction was not one of identity but rather one of cross reaction. These observations indicate that new or altered antigenic sites may be exposed when the molecule is dissociated into its subunits. Experiments are in progress to determine whether a specific subunit or group of subunits is responsible for the antigenic properties.

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Radiation Microbiology and Microbial Genetics

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6.1 NEW EXAMPLES OF UNUSUALLY RADIATION SENSITIVE MUTANTS OF *ESCHERICHIA COLI*

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Escherichia coli B_{s-1} was isolated from an irradiated culture of *E. coli* B and is remarkably sensitive to both ultraviolet and ionizing radiation.[1] The genetic characterization of this mutant has proven difficult because *E. coli* B and strains derived from it do not yield readily interpretable data when mated with the *E. coli* K-12 donor strains usually used for genetic mapping experiments. For this reason we thought it would be worthwhile to isolate strains of *E. coli* K-12 with radiation sensitivities similar to that of *E. coli* B_{s-1}. Such strains should allow more detailed genetic analysis of the factors controlling extreme radiation sensitivity. A culture of *E. coli* K-12 1899NM, an organism with many of the radiobiological characteristics of *E. coli* B, was exposed to a dose of radiation from a General Electric germicidal lamp sufficient to inactivate to the 10^{-6} survival level. Surviving cells were allowed to produce colonies on nutrient agar, and cultures derived from these colonies were screened for sensitivity to ultraviolet (2650 A) radiation. Four sensitive mutants were found among 300 cultures examined. Two of these (AB1899NM-114 and AB1899NM-294) have been studied in some detail, and survival curves are shown in Fig. 6.1.1. It can be seen that these K-12 mutants are approximately as sensitive as B_{s-1} to ultraviolet radiation (2650 A). These mutants are effective genetic recipients, and crosses will be performed to establish the genetic loci involved. They will also be examined for x-ray sensitivity, ability to excise uv-induced thymine dimers, and other radiobiological properties possessed by B_{s-1}.

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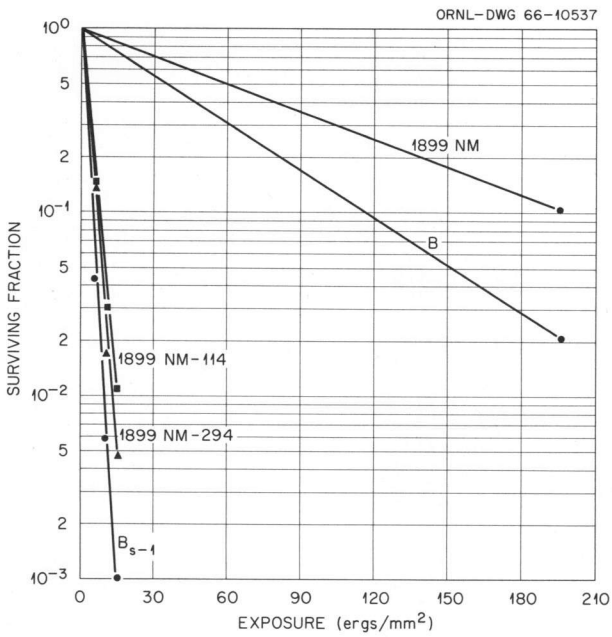

Fig. 6.1.1. Sensitivity of *E. coli* Strains to Ultraviolet Radiation (2650 A).

6.2 ISOLATION AND CHARACTERIZATION OF X-RAY-RESISTANT, UV-SENSITIVE STRAINS OF *ESCHERICHIA COLI*

H. I. Adler Alice A. Hardigree

Many mutants of *E. coli* with altered radiation response have been isolated and genetically characterized in recent years. Most of these novel strains are unusually sensitive to uv (2650 A) radiation. Some of them display, in addition, an increase in sensitivity to ionizing radiation. We have recently been successful in deriving a different class of mutants. These organisms are unusually resistant to ionizing radiation but have not undergone any significant alteration in resistance to ultraviolet light. To our knowledge, only one previous example of a similar, but not identical, mutation is available.[1] Such mutants may help us understand classes of damage produced by ionizing but not by ultraviolet radiation.

One of these x-ray-resistant mutants (*E. coli* P678-54), isolated after treatment of *E. coli* P676 with triethylmelamine, is being studied in detail. It has retained all the auxotrophic genetic markers of its parental culture, *E. coli* P678. It behaves normally when used as a recipient in crosses with *E. coli* K-12 HfrH, and tests are under way to determine the genetic locus (*i*) responsible for enhanced resistance to ionizing radiation. The only striking feature of the mutant, other than its radiation resistance, is its unusual behavior during growth and division. We have followed this process by time-lapse photographic techniques and have observed that many cells of this culture release small, roughly spherical structures from one or both and before normal cell division takes place. The mini-cells have not been observed to undergo any further growth or division but do seem to have the structure of small cells as judged by phase and electron microscope techniques. William Fisher, of the Molecular Physiology Group, has helped us obtain pure suspensions of mini-cells by sucrose density gradient centrifugation. We will next perform chemical analysis on mini-cells and normal cells in order to see if we can establish any connection between radiation resistance and this unusual division mechanism.

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6.3 GENETICS OF A RADIATION-RESISTANT MUTANT OF *ESCHERICHIA COLI* K-12

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We have succeeded in establishing the existence of two genetic factors responsible for the radiation resistance of the mutant R-5 of *Escherichia coli* K-12.[1,2]

One factor is the fertility episome F. It has been demonstrated that the relative x-ray resistance of isogenic lines derived from Hfr R-5 is Hfr $> F^- > F^+$ and that the transfer of F from Hfr to F^- cells by conjugation renders the recombinants resistant.

The other factor seems to be a chromosomal gene near a locus controlling arginine synthesis. X-ray resistance is found as an unselected phenotype among 35% (53/150) of the F^- recombinant for the arginine locus. The x-ray resistance gene is closely linked to, or identical with, a gene controlling sensitivity to leucine.

Our previous difficulty in obtaining resistant recombinants can now be explained by the observations (1) that two genetic factors influence radiation resistance in R-5 and (2) that the techniques previously employed would select against leucine-sensitive (x-ray-resistant) recombinants.

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6.4 INDUCTION OF CELL DIVISION IN RADIATION-INDUCED *ESCHERICHIA COLI* FILAMENTS

Amikam Cohen H. I. Adler

We are engaged in an attempt to characterize the cell-division-promoting system isolated from *Escherichia coli*.[1] This work is being carried out in collaboration with W. D. Fisher and G. E. Stapleton, of the General Physiology Group. The enzyme fraction of this system, P_2, is being purified by means of zonal ultracentrifugation, and the purified fractions are being tested for activity. Cell-division-promoting activity can be found only in 100S particles. We are currently examining various approaches to achieve further purification.

In addition, other division-inducing agents are being investigated. It has been found that in addition to pantoyl lactone, reduced glutathione and mercaptoethanol are division-promoting agents in radiation-induced filaments. The activities of these SH compounds approximate 2.5×10^3 division index units per mole.

Independently, attempts are being made to compare the properties of filament cell walls to normal cell walls. It has been found that the filaments of *E. coli* 1899NM are more sensitive to lysozyme digestion than nonfilamentous cells. This may indicate a significant difference in structure of the cell walls. Studies on differences in phage adsorption, sensitivity of cell walls to proteolitic enzymes, and difference in chemical structure are being conducted.

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6.5 EVOLUTION AMONG SUBSTRAINS OF *ESCHERICHIA COLI* K-12

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During the past several years we have made several comprehensive studies on certain aspects of the genetic properties of *Escherichia coli* K-12. The results of these studies indicate that a great deal of evolutionary divergence has occurred among the substrains used in our laboratory. In 1945, Lederberg and Tatum chose *E. coli* K-12 for their pioneer work on bacterial conjugation. The original K-12 strain was prototrophic, and by 1953 the five substrains which we have employed were already developed. These substrains are W1485 (F^+ λ^- prototroph), 58-161 (F^+ λ^+ *met*$^-$), K-12-112 (F^+ λ^+ *his*$^-$), W945 (F^- *thr*$^-$ *leu*$^-$ *thi*$^-$ λ^+ and carrying six fermentation deficiencies), and C600 (F^- *thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$).

The first indication of evolutionary divergence arose when we attempted to prepare isogenic donor and recipient strains from C600 and W945 derivations. We discovered that introduction of the fertility factor F into C600 and W945 to yield low-frequency F^+ donors did not allow us to isolate high-frequency Hfr donors. The transition from the F^+ to the Hfr state, which involves integration of F into the chromosome, did occur with measurable frequencies in the 58-161, W1485, and K-12-112 substrains. We have proven that the inability of F to integrate into the chromosomes of C600 and W945 is not due to differences in F, the presence or absence of λ, to any known genetic markers, or to environmental conditions. At present we still do not know the basis for this phenomenon. We can only surmise that the existence of C600 and W945 as F^- recipients over many generations has allowed them to lose whatever traits are necessary to become Hfr donors.

From the F^+ substrains W1485, 58-161, and K-12-112, which do give rise to Hfr donors, 70 Hfr donors have been isolated and characterized by us and by others. There are 23 sites at which

F has integrated into the bacterial chromosome. Integration of F at any site requires similar base sequences between F and the site in question to permit the reciprocal exchange necessary to integrate F. There are five such sites unique for F integration in K-12-112, seven unique sites in W1485, and five unique sites in 58-161. Sufficient numbers of Hfr strains have not yet been isolated to prove that these sites are unique in each strain. However, in one striking example we have nine independent Hfr strains from W1485 with F integrated at the same site and no examples of F integration at this site in either 58-161 or K-12-112. Furthermore, there are only two sites on the bacterial chromosome which permitted F integration with the formation of Hfr donors in all three F^+ substrains. Therefore, we have concluded that as a consequence of evolutionary divergence, changes in base sequence in F or in the bacterial chromosome have occurred in these three substrains to add or subtract F integration sites which did or did not exist in the original K-12 parent.

A third instance of evolutionary divergence was uncovered during routine mapping experiments using the interrupted mating technique. Hfr strains derived from 58-161 transfer the interval between the ade_3 and *try* loci in 9 min and the interval between *try* and *his* in 14 min. Hfr strains derived from K-12-112 also transfer the interval between ade_3 and *try* in 9 min but take 25 min to transfer the *try* to *his* interval. Hfr strains from W1485, however, take 22 min to transfer the ade_3 to *try* segment and 25 min to transfer the *try* to *his* interval. These differences in rates of chromosome transfer were the same regardless of the derivation of the F^- recipient parent. We have shown that the increase in transfer time for the ade_3 to *try* segment by W1485 Hfr strains is not due to transpositions or to other chromosomal rearrangements. By using the *gal* marker, which is between ade_3 and *try*, it has been shown that the interval between ade_3 and *gal* is transferred in 2.5 min by 58-161 Hfr strains and in 6 min by W1485 Hfr strains. The ratios 2.5:9 for 58-161 and 6:22 for W1485 yield nearly identical values of 0.28 and 0.27, indicating that the rates of chromosome transfer in the ade_3 to *try* segment are proportionally slower for W1485 Hfr strains. Of interest is the fact that the transfer time for the *leu* to ade_3 segment is an identical 13.5 min for Hfr strains derived from all three F^+ substrains. Complete mapping of the entire chromosome for each of the three substrains has not yet been finished, although we already have experiments with Hfr strains from W1485 which show that transfer times for intervals in other parts of the chromosome are longer than published values.

A fourth instance of evolutionary divergence became apparent during studies on the isolation of pro^- mutants. We had previously discovered that the three genes coding for the enzymes in proline biosynthesis were not contiguous in *E. coli* as they are in *Salmonella*. We also developed rapid methods employing Hfr strains and cross-feeding tests for localizing any given pro^- mutation to one of the three *pro* cistrons. Thus, we have isolated over 1000 pro^- mutants from various substrains of *E. coli* K-12. Derivatives of W1485 give only pro_1^- mutants, derivatives of W945 give only pro_1^- and pro_3^- mutants, derivatives of 58-161 give only pro_2^- and pro_3^- mutants, while derivatives of C600 give rise to pro^- mutants for all three *pro* cistrons. To explain the absence of pro^- mutants for a given cistron, we postulated that that cistron was duplicated in that substrain. Preliminary data from hybrid strains prepared by transduction give some support to this idea. In any event it is obvious that evolutionary change has occurred in regard to specific mutability of the *pro* cistrons in each of the K-12 substrains so far studied.

The above four examples of evolutionary divergence in *E. coli* K-12 are most convincing. Our other studies on the control of chromosome replication during vegetative growth, on the role of the F^- parent during bacterial conjugation, and on the requirements for energy metabolism and DNA synthesis during chromosome transfer also show evidence of evolutionary change which can only be substantiated by further work. The evidence so far accrued, however, strongly favors our contention that meaningful conclusions can only be drawn after careful study employing isogenic strains. Likewise, it would appear profitable to use *E. coli* K-12 as a test organism for studying evolutionary events in the laboratory. Its rapid growth and the ability to maintain continuously growing cultures would facilitate such studies.

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Radiation Immunology

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7.1 GROWTH AND DECLINE OF COLONY-FORMING CELLS IN THE SPLEENS OF NEONATAL MICE

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Introduction. – It has been shown[1] that the progenitors of antibody-forming cells increase in the spleens of neonatal and juvenile mice at a rate that appears to be considerably greater than the overall rate of growth of the organ. Such progenitor cells are considered to be pluripotential. It was desirable to learn whether or not colony-forming cells (CFU), which are pluripotential progenitor cells of the hemopoietic systems, displayed similar allometric growth in neonatal animals.

Methods and Results. – Mice of the BC_3F_1 (C57BL ♀ × C3H ♂) strain were employed. Recipient animals were 12 to 16 weeks of age and were exposed to 700 r of x rays. Into such recipients, varying numbers of spleen cells were infused from donors ranging in age from 5 days to 40 weeks. After nine days of culture, recipient mice were sacrificed and their spleens removed and fixed in Bouin's fluid. Easily discernible macroscopic colonies were counted. There was a linear relationship between the number of macroscopic colonies and the number of infused donor cells taken from donors of all ages over the range in which colony counts were feasible. The results showed that the frequency of CFU is highest in the youngest animals examined and declines with advancing age. The rate of increase in CFU per organ during the first five weeks of age is consonant with the rate of growth of the spleen during this period. The fall in number of CFU per spleen between 5 and 12 weeks of age is unrelated to

overall change in organ weight or to total number of cells per spleen.

Discussion. – It will be important to learn from further studies the significance of the peak in frequency of CFU in the spleen of newborn mice and of the decline in subsequent weeks. This may reflect the dominance for hematopoiesis of, first, the yolk sac and then, sequentially, liver, spleen, and bone marrow during ontogenesis. If true, the hematopoietic activity of the bone marrow may depend upon initial "seeding" by progenitor cells from the spleen.

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7.2 FURTHER STUDIES ON THE SECONDARY ANTIBODY-FORMING POTENTIAL OF JUVENILE, ADULT, AND AGED MICE

Takashi Makinodan W. J. Peterson

Introduction. – In previous reports[1] it was shown that the secondary antibody-forming potential (SAFP) of mice increases rapidly after antigenic priming reaches a maximum and thereafter decays exponentially with advancing age at a rate comparable to that of the primary antibody-forming potential (PAFP). In an attempt to gain better insight into the causes of variation with age in the SAFP per mouse and spleen, further studies were carried out with mice of varying age groups.

Results. – The following results were obtained. (1) The SAFP per spleen within any age group of mice, except for the very old, was greater among donors with persisting primary serum antibody titer than among those without, but this relationship was not quantitative nor was it apparent between donors of different age groups. (2) Among the very old mice the SAFP *per mouse* was as low as the PAFP per mouse, but the SAFP *per spleen* was greater than the PAFP per spleen. (3) Decrease in capacity of very old mice to synthesize mercaptoethanol (ME)-resistant antibody was more pronounced than decrease in the capacity to synthesize ME-sensitive antibody.

Discussion. – These results indicate that the homeostatic factors responsible for decrease in the relative number of progenitor cells with age do not show any preference between those of previously immunized and nonimmunized individuals. Furthermore, they indicate that during senescence the physiological environment is aging in addition to the functional lymphoidal tissue. Finally, the data suggest that during senescence the immunologic potential is gradually reverting to its embryonic and neonatal level. Hence, there is a need to study the role of juvenile and aged physiological environments on immunologically competent cells derived from juvenile and aged donors.

References

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7.3 DEVELOPMENT OF IMMUNOLOGIC COMPETENCE IN GERMFREE AND CONVENTIONAL MICE

M. J. Bosma H. E. Walburg, Jr.[1]

Introduction. – It has been shown in previous studies[2] that the ability or capacity of an individual to form antibody (Ab), although influenced by regulatory factors, is a function of the "pool size" of immunocompetent progenitor cells, the size of which increases with age. The present report is concerned with the role of environmental factors on the developmental aspects of these cells as reflected by changes in the immune potential.

Methods and Results. – The immune potential of germfree (GF) and conventional (CVN) mice was assessed at the cellular level by transferring known numbers of dispersed spleen cells from mice of various ages together with an optimum concentration of the test antigen (sheep red blood cells) into lethally irradiated, and therefore immunologically unresponsive, recipient mice. In this system the primary Ab-forming potential (PAFP) of the transferred spleen cells is expressed in terms of the serum hemagglutinin titer of the plateau phase of response. It was found that the kinetics of Ab expression for GF and CVN spleen cells were the same at all ages tested and that

the PAFP of GF and CVN spleens described typical growth curves which were identical. The PAFP of the spleen developed exponentially during the first five weeks after birth, with a doubling time of about four days, and then gradually to a maximum plateau level at nine weeks. During this period of growth the PAFP of the spleen grew approximately 500-fold. The growth of the spleen was substantially less. During the first five weeks after birth, it doubled every two weeks and after nine weeks revealed only about a fourfold increase. The immune competence of GF and CVN mice, as estimated by the pool size of immunocompetent progenitor cells, was found to be equivalent at 3 and 13 weeks of age (5–10 and 45–70 immunocompetent progenitor cells respectively).

Discussion. – Since the essence of GF research is isolation, GF mice, unlike CVN mice, are not exposed to an uncontrollable influx of antigenic stimuli. These results show that in the GF state the relative absence of "naturally occurring antigens" (other than dietary antigens) is of no consequence to the functional and developmental aspects of the immune potential. That is, an exposure to living microbes is not a prerequisite for the expression of a "normal" immune potential, and immunological competence is possibly more a function of chronological age than it is of the environment.

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7.4 SIGNIFICANCE OF A SINGLE-HIT EVENT IN THE INITIATION OF ANTIBODY RESPONSE

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J. F. Albright

Introduction. – The origin of the physiochemical and immunochemical heterogeneity of immunoglobulin molecules has been a perplexing problem for many years. One approach to this problem is an analysis of immunoglobulins at various times during response by the progeny of a single precursor cell. Before such clonal studies can be attempted, it is obviously necessary to have some clear notion about the number and frequency of these precursor cells.

Results and Discussion. – The enumeration of precursor cells is best done in a transfer system in which dispersed spleen cells are cultured with an optimum dose of antigen in a mouse rendered immunologically incompetent by prior x irradiation.[1] The amount of antibody formed in such a system is a linear function of the number of cells transferred.[2] This observation strongly suggests that there are independent immunocompetent units which control antibody formation. If there are independent units which control the production of antibody, it should be possible to reduce the inoculum size so that there is a finite probability of the inoculum containing only one or more of the controlling units. Statistical methods based on the Poisson distribution would be applicable to such an experiment:

$$\ln p_0 = -n \,, \qquad (1)$$

where p_0 is the probability of a null response and n is the number of immunological units.

In a series of experiments using rat red blood cells as the antigen, the frequency of nonresponders as a function of inoculum size was determined for nonprimed and primed donors. The number of units in the spleen cell inoculum was calculated using the statistical method of Finney,[3] and the frequency of observed nonresponders as a function of the number of immunological units was compared with that predicted by Eq. (1). As shown in Fig. 7.4.1, a comparison of frequencies predicted by Eq. (1) with observed frequencies, gave a chi square[4] of 230 with 35 degrees of freedom. The probability of observing this good a fit or better by chance would be 0.05, and one concludes that there are independent units controlling the synthesis of hemagglutinating antibody, regardless of whether the response is primary or secondary. Although it remains to be proved, it is very reasonable to surmise that the independent immunocompetent unit is the precursor cell for the functional antibody-producing cells.

These statistical methods should be useful in analysis of the peptides produced by functional cells in the immune system.

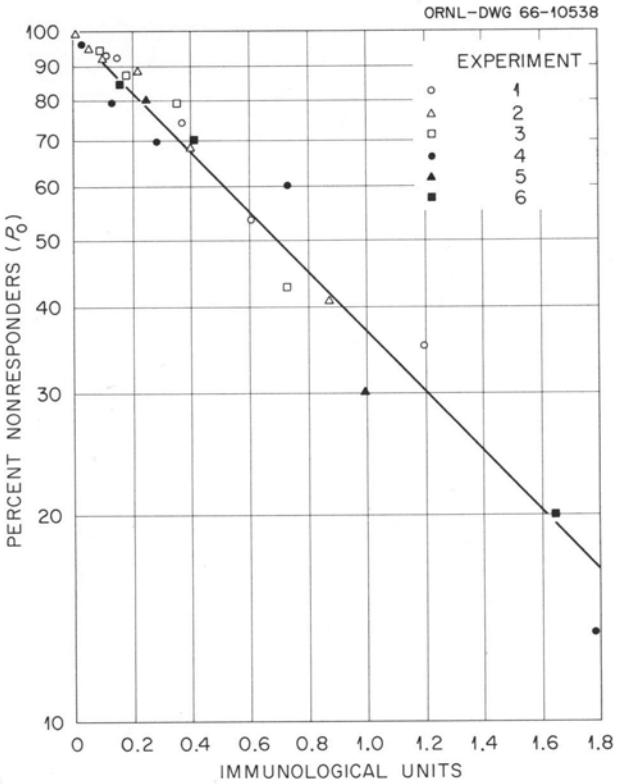

Fig. 7.4.1. Frequency of Nonresponders as a Function of Immunological Units.

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7.5 PRODUCTION OF 7-S AND 19-S ANTIBODIES IN THE MOUSE BY A LIMITED NUMBER OF PROGENITOR CELLS

A. E. Sassen

Introduction. – The problem of heterogeneity of specific antibodies and antibody-synthesizing cells can never be resolved unless a method can be perfected which can accurately detect antibodies and functional progeny of a single antigen-stimulated progenitor cell. To obtain this objective, attempts were made to culture spleen cells with antigen in vitro and in vivo.

Results. – Initially, many attempts were made to perfect culture and antibody-detecting methods. It was decided to use the in vivo culture (cell transfer) method in which lethally irradiated recipients are injected with a mixture of spleen cells from primed and nonprimed mice together with the test antigen. Using rat red blood cells (RBC) as the test antigen, the following results were obtained. (1) During the early phase of secondary response by a limited number of primed spleen cells, only mercaptoethanol (ME)-sensitive 7-S antibodies were detected, and one week after culture, only ME-resistant 7-S antibodies were detected. (2) During early primary response, only ME-sensitive 19-S antibodies were detected, but ME-resistant 7-S antibodies were also detected one week after culture. (3) In terms of efficiency of the primary vs secondary responses, a minimum of 10^7 and 10^5 spleen cells, respectively, were needed to produce detectable levels of antibody during the first week after culture.

Discussion. – These results indicate that progeny of primed progenitor cells are distinct from those of unprimed progenitor cells. More comprehensive studies are in progress to further characterize the types of antibodies synthesized early and late in the immune response after culture of a limited number of spleen cells with RBC and protein antigens.

7.6 PROTEIN SYNTHESIS IN ANTIBODY-FORMING CELLS

Toshiteru Morita R. A. Brown

Introduction. – Previous studies have shown that polyribosomes containing five to ten ribosomal units in antibody-forming cells were obtained under the condition where the activity of ribonuclease was minimized by using the supernatant of regenerating mouse liver extract and polyoxyethylene acetyl ether during the homogenization of spleen cells.[1] However, these techniques

were not adequate for obtaining sufficient quantities of the large polyribosomal units from the antibody-forming cells. Using the whole immunized spleen, the principal peak observed by optical analysis and ^{14}C-amino acid incorporation was still in the monoribosome region. Therefore purified cultures of antibody-producing cells obtained through the use of diffusion chambers and high doses of x-ray irradiation were used for the polyribosome preparations.

Methods and Results. – Chambers containing 24×10^6 primed spleen cells and the test antigen were implanted into the peritoneal cavity of sublethally irradiated isologous recipients. On day 6 after culture the recipients were irradiated (5000 r), and the chambers were removed from the irradiated recipients. The irradiated chambers were reimplanted into another sublethally irradiated isologous recipient. Three days after the transfer the chambers were removed from the recipients, and cells were collected from the chambers after treatment of pronase.[2] The results of incorporation of ^{14}C-amino acids and autoradiograms of immunoelectrophoresis showed that the cells, which were irradiated by very high doses of x ray and treated with pronase, synthesized mainly gamma globulin in vitro. Polyribosomes were extracted from diffusion chamber cells which had been irradiated with 5000 r and treated with pronase. The optical pattern and the incorporation of ^{14}C-amino acids showed that the main peak was still in the monosomal region, but significant peaks were also found in the region of three to ten ribosomal units.

Discussion. – The fact that the heavily irradiated antibody-forming cells can synthesize gamma globulin would suggest that the antibody-synthesizing mechanism may be quite stable and that the messenger RNA for antibody synthesis may have a long half-life.

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7.7 ENGULFING POTENTIAL OF PERITONEAL PHAGOCYTES FROM CONVENTIONAL AND GERMFREE MICE

E. H. Perkins
Toshiteru Morita
Paul Nettesheim[1]
H. E. Walburg, Jr.[2]
Glenda A. Davis

Introduction. – It is not known what role naturally occurring microbial flora play in the development of the phagocytic capacity of peritoneal phagocytes. Quantitative and comparative studies of phagocytes for conventional and germfree animals are therefore desirable. With the development of a simple quantitative method[3] we have been able to measure the in vitro engulfing potential of peritoneal exudate cells from different strains of mice raised in conventional or germfree environments.

Results. – Peritoneal exudate cells harvested from both conventional and germfree mice which had received a prior intraperitoneal injection of a sterile irritant (gelatin) possessed markedly enhanced engulfing capacities. The percentage of identifiable histiomonocytic cells in these stimulated exudates was always greater in mice raised in a conventional environment. Although strain differences were apparent, no difference could be detected in the engulfing potential of the phagocytic cells from conventional and germfree mice of the same strain. Peritoneal phagocytes from ICR had a greater phagocytic potential than C_3H mice, which, in turn, had a greater phagocytic potential than CF No. 1 mice. This enhanced phagocytic potential was shown to be due to the functional maturation of histiomonocytic cells and not to transformation of nonhistiomonocytic cells into phagocytic elements.

Discussion. – These results show that naturally occurring microbial flora is unimportant in the development of the engulfing potential of peritoneal phagocytes. Whether or not it plays a significant role in the development of the engulfing potential of phagocytes of the respiratory tract and other parts of the body remains to be determined.

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Mammalian Genetics

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Overall Measures of Genetic Damage (8.2)

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Effects of Radiation on Mammalian Gametogenesis (8.5)

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Participation in Cooperative Programs:
AEC-NIH Chemical Carcinogenesis (22.19)

8.1 MUTATION FREQUENCY IN FEMALE MICE EXPOSED TO HIGH-INTENSITY X IRRADIATION DELIVERED IN SMALL FRACTIONS

W. L. Russell Elizabeth M. Kelly

An earlier report[1] presented data showing that the mutation frequency in female mice exposed to a small x-ray dose (50 r) at high dose rate was lower than expected on the assumption of a straight-line relationship with the mutation frequencies from large doses at high dose rate. This finding is obviously of great importance in the estimation of genetic hazards, and an attempt has been made to check it in an experiment designed to avoid the vast numbers of animals required to establish a reliable mutation frequency with a dose as low as 50 r. In this new experiment a total dose of 400 r of 90-r/min x irradiation was given to female mice in eight fractions of 50 r spaced 75 min apart. The rationale was that, since females exposed to a continuous dose

Table 8.1.1. Mutation Frequency in Conceptions Occurring in the First Three Weeks After Irradiation of Female Mice with 90 r/min X Rays

Total Dose	Fractionation	Number of Offspring	Number of Mutations at 7 Loci
400 r	8 × 50 r at 75-min intervals	23,387	13

of 400 r of 0.8-r/min irradiation give a reduced mutation frequency compared to that from 90-r/min irradiation, perhaps 50-r fractions of 90-r/min irradiation distributed over the same total time of exposure as that required in the 0.8-r/min irradiation might also give a low mutation frequency.

The results obtained for conceptions occurring within the first three weeks after irradiation are shown in Table 8.1.1. Mutations were scored by our standard specific-locus method. The data were restricted to the three-week interval for comparison with the data from single 400-r exposures which were similarly restricted. The observed number of mutations, 13, is significantly ($P = 0.001$) below the approximately 36 that would have been expected on the basis of the frequency obtained from single 400-r exposures (32 mutations in 20,927 offspring).

Thus the fractionation experiment confirms the finding from the 50-r single-dose experiment that small doses of high-dose-rate irradiation give fewer mutations than would be expected on the basis of a straight-line relationship with the mutation frequency from large doses at the same dose rate.

The results indicate that some repair of mutational or premutational damage can occur at low doses as well as at low dose rates.

In estimating human genetic hazards it would now appear that the risk from small doses of acute irradiation may, in females at least, be lower than had been estimated on the basis of large doses.

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8.2 EFFECT OF BREEDING ON THE REPRODUCTIVE CAPACITY OF IRRADIATED FEMALE MICE

U. H. Ehling Sandra W. Huff

It was reported earlier that the reproductive capacity of irradiated females is affected by a chromosomal and a maternal factor.[1] In these experiments virgin females, 10 to 12 weeks old, were irradiated with 50 r and mated to C3H males.[2] In order to analyze the chromosomal factor, backcross females of the following crosses [SEC × (SEC × DBA)F_1] were allowed to produce one litter before irradiation. The mean litter size in successive litters of these females and females of the same genetic constitution, but irradiated as virgin females, is summarized in Table 8.2.1.

In the unirradiated controls the litter size increased from the first to the fifth litter and then decreased. The mean number of litters for unirradiated females is 14.2. In the irradiated groups the first litter was the largest. Very few females produced three or more litters. In contrast to irradiated virgin females, the group of females mated before irradiation showed a pronounced increase in litter size. The large first litter is due to superovulation.[3] Based on the age of the irradiated females, the first litter of the 50-r group with one litter before irradiation corresponds to the third litter in the control group.

The cause of the 42% increase in number of offspring per female in the group of females with one litter prior to irradiation as compared to females without litters prior to irradiation is not yet known. It is, however, unlikely that the difference between the irradiated groups is due to different ages of females at the time of irradiation.[4] Further experiments are planned to analyze this observation because of the general interest of this factor in regard to studies of radiation-induced mutations. The effect of breeding on the reproductive capacity of the irradiated female mice is of particular interest in experiments where differences in the mutation rate were observed between irradiated young virgin females and older females with litters before irradiation.[5] This factor may also have implications for studies where the interval between irradiation and conception has a striking effect on the mutation frequency seen in the offspring.[6]

Table 8.2.1. Comparison of Litter Size of Irradiated and Control Females

Treatment	Number of Fertile Females	Mean Litter Size in Successive Postirradiation Litters						
		1st	2d	3d	4th	5th	6th	7th
Zero litter + 0 r	26	6.3	6.4	7.7	7.7	8.8	8.5	7.5
Zero litter + 50 r	51	5.8	4.8	0.7	0.2	0.1	0	0
One litter + 50 r	38	9.3	6.5	0.5	0.2	0	0	0

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8.3 DEFICIENCIES IN THE MOUSE

Liane B. Russell Mary Sue Steele
H. M. Thompson, Jr.

Introduction. – Two of the markers used in specific-locus mutation-rate experiments are *d* (dilution) and *se* (short-ear), which give only 0.16% recombination (one of the closest linkages known in the mouse). Double mutants (*d se*) are presumed to carry deficiencies. In experiments in which spermatogonia are irradiated with single, acute doses of x rays, or with protracted doses of gamma rays, *d se* mutants are found very rarely in comparison to single *d* or *se* mutants. The treatment of other cell stages or the use of other types of irradiation has, however, yielded higher proportions of *d se* mutants. Part of a study involving 33 of these, derived from a variety of experiments, is summarized here. Tests are still in progress for three additional *d se* mutants. Omitted are six *d se*'s which, on the basis of various lines of evidence,[1] were concluded not to be deficiencies but the result of nondisjunction in both parents. Five of these occurred in controls.

Methods and Results. – The mutants in this study were mated to wild type, and their offspring – which were presumably ++/*d se* (i.e., containing the test-stock markers) or ++/Def – were individually mated to d^{op}+/*d se*. The symbol d^{op} designates any of a number of mutations at the *d* locus that give opisthotonic convulsions either in homozygous condition or in the d^{op}/Def combination. Thus, individual F_1 offspring of the *d se* mutants were classified as ++/Def or ++/*d se*, respectively, depending on whether the dilute long-eared class in their progeny (F_2) did or did not have convulsions. A total of 1544 F_1's were tested (yielding many thousands of test-cross progeny). In Table 8.3.1, mutants are listed in decreasing order of proportion of ++/Def progeny, as established by this test mating. Also listed (in the adjacent column) is a maximum estimate, derived by including also those F_1's (a total of 201 for all mutants) which were untestable because they died young, were sterile, or produced insufficient progeny to include a dilute long-eared class. For the purposes of the maximum estimate, these are assumed to be ++/Def. The differential between the two columns gives some measure of heterozygous deleterious effects of the deficiencies in *post*natal life. Any significant depression from 50% in the maximum estimate indicates either *pre*natal mortality of some of the ++/Def progeny or reduced transmission of gametes carrying the deficient chromosome.[1]

Inspection of Table 8.3.1 indicates a wide spread among the mutants with respect to proportion of ++/Def in their progeny. For the first 16 this

Table 8.3.1. Deficiencies in the *d se* Region of the Mouse: Induction Factors, Progeny Ratio, Extent of Deficiency

Mutants are ranked by proportion of deficiency-bearing F_1, as established by progeny tests[a]

Mutant				Irradiation Data				F_1				
Symbol and Sex			Size	Germ Cell Stage Irradiated	Type of Irradiation	Total Dose[b]	Dose Rate (rads/min)	Number Tested	Number Classified	Percent with Deficiency Among Tested	Maximum Estimate[c]	Does Deficiency Include *sv*?
FATw	♂	39	Normal?	Oocytes	X	200	80–100	65	67	63.1	64.2	No
DTD	♀	4	Normal	Spermatogonia	X	500 + 500	80–100	46	47	60.9	61.7	No
RD300H	♀	1	Normal	Spermatogonia	Neutron	74 + 155	80–100	52	56	59.6	62.5	No
CoS	♀	43	Normal	Spermatogonia	Gamma	600	24	79	86	59.5	62.4	No
R60L	♀	13	Normal?	Spermatogonia	Neutron	63	0.16–0.2	42	47	52.4	57.4	No
G	♀	130	Normal?	Sperm or 'tids	X	300	80–100	6	7	(50.0)	(57.1)	No
R145L	♂	11	Sl. small	Spermatogonia	Neutron	115	0.16–0.2	76	79	50.0	51.9	No
FR60L	♂	8	Normal?	Oocytes	Neutron	62	0.16–0.2	72	75	47.2	49.3	No
FBFo	♂	37	Normal	Oocytes	Gamma	400	0.8–1.0	74	79	45.9	50.6	No
RD300H	♂	4	Sl. small	Spermatogonia	Neutron	74 + 155	80–100	68	76	45.6	51.3	No
SaSd	♂	13	Small	Spermatogonia	X	600	8–10	51	56	45.1	50.0	No
DTD	♀	1	Normal?	Sperm	X	500 + 500	80–100	63	68	44.4	48.5	No
R75M	♀	20	Normal?	Spermatogonia	Neutron	59	0.8–1.0	67	70	43.3	45.7	No
Z_b	♂	17	Small	Oocytes	X	400	80–100	73	87	42.5	51.7	No
$FATW_b$	♂	18	Sl. small	Oocytes	X	200	80–100	65	70	41.5	45.7	No
R250M	♀	2	Sl. small	Spermatogonia	Neutron	202	0.8–1.0	73	97	41.1	55.7	No
DTW	♂	24	Sl. small	Spermatogonia	X	500 + 500	80–100	66	73	37.9	43.8	No
G	♂	209	Small	Sperm or 'tids	X	300	80–100	59	68	37.3	45.6	Yes
SAS	♂	3	Sl. small	Sperm	X	600	8–10	77	85	36.4	42.4	No
R145H	♂	1	V. small	Sperm	Neutron	105	80–100	74	79	35.1	38.8	No
G	♀	99	Small	Sperm	X	300	80–100	35	40	34.3	42.5	No
FBFo	♀	94	Normal?	Oocytes	Gamma	400	0.8–1.0	42	45	33.3	37.8	No
FDFoD	♀	10	Sl. small	Oocytes	X	200 + 200	80–100	40	50	27.5	42.0	*d*
DTD	♂	7	Small	Spermatogonia	X	500 + 500	80–100	23	27	26.1	37.0	*d*
R75H	♀	1	Small	Spermatocytes	Neutron	59	80–100	33	51	21.2	49.0	Yes
Z_b	♂	8	Small	Oocytes	X	400	80–100	33	46	15.2	37.8	
CoS	♀	2	Small	Sperm	Gamma	600	24	19	23	10.5	26.1	
R75H	♀	3	Normal?	Spermatogonia	Neutron	59	80–100	19	29	5.3	37.9	Yes
Z_b	♂	23	Sl. small	Oocytes	X	400	80–100	6	8	(0)	(25.0)	
FR120H	♀	16	V. small	Oocytes	Neutron	124	80–100	14	18	0	22.2	
FATw	♀	8	Small	Oocytes	X	200	80–100	11	14	0	21.4	
G	♀	201	Small	Sperm	X	300	80–100	21	22	0	13.6	
G	♂	200	Small	Sperm	X	300	80–100	Sterile mutant				

[a]Excludes double nondisjunction types.

[b]Roentgens for x and gamma rays, rads for neutrons. For neutrons, the doses to the gonads are estimated to be somewhat lower than those listed, and the figures given include a gamma component equal to approximately one-seventh of the neutron component.

[c]Includes: died young; sterile; and test mated but not producing diagnostic type (too few young).

[d]Def/*sv* heterozygotes do not waltz but are very small.

does not differ significantly from 50%; the remaining mutants seem to fall into two or three groupings. Earlier tests had indicated that even those deficiencies that are in the top group are homozygous lethal at a very early stage. It may be noted that the size of a mutant (relative to its littermates) seems to be broadly correlated with its rank in the table. Thus, among the first 16, 10 are normal; but among the remaining 17, only 2 are normal.

As stated above, *d se* deficiencies are not commonly induced in spermatogonia except by treatment with neutrons. Those *d se*'s that are induced in spermatogonia seem, in general, to be less deleterious than those induced in postgonial stages (spermatocytes, spermatids, spermatozoa). Thus, spermatogonial *d se* mutants are distributed between the top group and all the more deleterious groups in the ratio of 9:3; postgonial mutants, in the ratio of 2:9. Deficiencies induced in oocytes are spread randomly.

Of the 33 deficiencies listed in Table 8.3.1, 26 have been tested against *sv*, Snell's waltzer, which gives about 2% recombination with *d se*.[2] The results are in the last column of the table. Unfortunately, many of the most deleterious of the deletions were not available for test, since in five of them no stocks could be obtained (no ++/Def surviving and fertile), and in two others the stocks were of such poor viability and fertility that they had been lost by the time *sv* was obtained. Three of the remaining deficiencies were found to include *sv*, that is, in the test mating *d* ++/Def x + *se sv*/+ *se sv* the short-eared progeny were waltzers and also small. These were tested with another marker, *tk* (tailkinks) (*d se* [2] *sv* [4] tk) but found not to include that locus. In the case of two other deficiencies, the short-eared progeny did not waltz but were quite small. The first 16 of the ranked deficiencies gave no evidence of extending even as far as *sv*. In addition to the 26 *d se* deficiencies, five *d* and four *se* prenatal lethals were also tested with *sv*, but found not to include it.

Conclusions. – Observations on deficiencies in the *d se* region of the mouse may be summarized as follows:

1. No spontaneous occurrences have been found.
2. All are homozygous lethal; but in heterozygous condition, they vary from being relatively innocuous to being quite deleterious in terms of viability and fertility.
3. Many show a heterozygous effect on size; this is particularly true of the more deleterious ones.
4. They are not commonly induced in spermatogonia, except under certain radiation conditions; those that are induced in spermatogonia seem, in general, to be less deleterious than those induced in postgonial stages.
5. Recovered deficiencies are relatively short in terms of crossover units involved, as judged by tests on one side of *d se*.

References

[1]Liane B. Russell and W. L. Russell, *J. Cell. Comp. Physiol., Suppl. 1* **56**, 169–88 (1960).

[2]Margaret C. Green, personal communication, 1965.

8.4 SEARCH FOR INTRASTRAIN AND INTERSTRAIN REARRANGEMENT HETEROZYGOSITY IN THE MOUSE

R. B. Cumming

We have initiated a program of screening the various inbred strains of mice maintained in our laboratory for possible chromosomal rearrangements carried in the laboratory populations. This project is to provide basic data necessary for a number of other cytogenetic studies currently in progress in the Mammalian Genetics section.

Males approximately two months old are selected from the inbred strains or from F_1 hybrids derived from crosses between different inbred strains. Testes are removed, and preparations are made using either a squash technique[1] or an air-drying technique involving a cell suspension.[2] The preparations are then scored for rearrangement heterozygosity. A minimum of 100 cells per animal are scored.

To date we have data on five inbred strains and F_1 hybrids between these five strains. The strains for which we now have data are 101, C3H, C57BL/10, A, and DBA. No intrastrain or interstrain rearrangement heterozygosity has shown up to date.

References

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[2]E. P. Evans, G. Breckon, and C. E. Ford, *Cytogenetics* **3**, 289 (1964).

8.5 SURVIVAL OF SPERMATOGONIA OF THE MOUSE AFTER IRRADIATION IN S AND G_2 STAGES OF THE MITOTIC CYCLE

E. F. Oakberg Evelyn C. Lorenz
Roselynn A. Dynesius

The possible contribution of differential cell survival to the decrease in mutation frequency at high acute doses and to the high mutation rate obtained with two 500-r exposures 24 hr apart[1] have been evaluated by studying the survival of mouse spermatogonia irradiated in S and G_2. Preliminary results indicated a higher survival of cells in G_2 after 1000 r, and the possibility that this may be a factor in the drop in mutation rate at high doses was suggested.[2] The more extensive data reported here, however, now make this interpretation unlikely.

Male mice (101 × C3H), approximately 12 weeks old, were used. Tritiated thymidine was injected either 30 min before irradiation, in order to score survival of spermatogonia irradiated in S, or 6 hr before irradiation, in order to label cells irradiated in G_2. Autoradiographs of 3-μ paraffin sections were prepared, and any cell with three or more silver grains above the nucleus was classified as labeled. Six hundred cells were scored for controls and for the 600-r series. All available cells were scored for 1000 and 500 + 500 r, and the totals ranged from 233 to 600 cells per treatment.

After irradiation, frequencies of labeled cells were much lower than in controls (Table 8.5.1). The lowest survival was for cells irradiated in S, and G_2 was slightly higher. The majority of surviving cells were unlabeled (i.e., irradiated in G_1 or late G_2). The contribution from late G_2 probably was negligible, since we previously have observed rapid degeneration of prophase stages after irradiation at high doses. Frequencies of labeled cells remained constant after five days, indicating that they actually were participating in repopulation of the testis.

Differences between treatments were small, but the lowest frequency of labeled cells for irradiation in S was after 600 r, with 1000 r and 500 + 500 r slightly higher. Survival of G_2 was lowest after 500 + 500 r, with 600 r and 1000 r showing somewhat higher proportions of labeled cells. All these differences are too small to explain the mutation rate data of Russell[1] on the basis of differential cell survival.

After accounting for total labeled cells by combining the survival of S and G_2, the remaining unlabeled cells, which were presumably in G_1 at the time of irradiation, constitute 75 to 85% of the surviving spermatogonia for all three doses. Thus, in all cases the majority of surviving cells are those expected to have the highest sensitivity to mutation if the data of Kimball[3] for *Paramecium* also apply to mouse spermatogonia. Intracellular mechanisms therefore are suggested as being of primary importance in the decrease in mutation frequency at high acute doses, and they also may be a factor in the enhanced effect of fractionated exposures.

Table 8.5.1. Percentage of ^{3}H-Thymidine-Labeled Spermatogonia After Irradiation in S and G_2

Time After X Ray (days)	S				G_2			
	0 r	600 r	1000 r	500 + 500 r	0 r	600 r	1000 r	500 + 500 r
	40.17				37.83			
3		5.67	5.49	6.83		6.00	5.00	8.96
5		5.67	9.12	10.30		12.00	11.31	6.82
6		6.97				14.14	13.17	9.00
8		7.67	10.00	11.49		12.00	16.44	12.46
10		8.83	10.46	5.95		11.83	13.17	8.61

Finally, it must be emphasized that the possibility of differential cell survival cannot be completely excluded by experiments of this nature. While labeled cells keep pace with the entire spermatogonial population during early recovery stages, it is not possible to follow individual cells to sperm production. Also, the contribution of differential survival of G_1, S, and G_2 appear to be ruled out by these experiments, but it is not possible to exclude the possibility of variation in radiation response within these stages.

References

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

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[3]R. F. Kimball, N. Gaither, and S. W. Perdue, *Intern. J. Radiation Biol.* **3**, 133 (1961).

Human Cytogenetics

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9.1 RELATIONSHIP OF INCOMPLETE CHROMATID ABERRATIONS AND THE CELL CYCLE

J. G. Brewen H. E. Luippold

Introduction. – The classical theory of aberration production by ionizing radiations has been seriously questioned in recent years by Revell.[1] Revell proposes that instead of an ionizing particle breaking the chromosome strand and these breaks remaining open or interacting to form aberrations, the ionizations cause a localized active lesion in the chromosome. Whenever two of these lesions come in contact an exchange of material occurs, and an aberration is formed. Inherent in this theory is that all aberrations are exchanges.

Revell has made calculations on the theoretical yields of the different types of intrachange that will give either a terminal deletion or isochromatid deletions. He has assumed that all types of exchange have an equal probability of occurring. Five types of incomplete exchange will give rise to a terminal deletion, whereas two types of incomplete exchange will give rise to incomplete isochromatid deletions. Since they all have an equal chance of occurring, the ratio of terminal deletions to incomplete isochromatid deletions should be 2.5:1. Revell, himself, has data that show such a ratio.

Materials and Methods. – Lateral roots of the broad bean, *Vicia faba,* were used. The roots were irradiated with 125 r of x rays generated by a G.E. Maxitron x-ray machine operated at 250 kvp and 30 ma with 3 mm of aluminum filtration. Immediately after irradiation, continuous 2-hr colchicine samples of metaphase chromosomes were made up to 12 hr after irradiation. A total of 300 aberrant cells were scored at each sampling time, wherever possible. Aberrations were classified as to their type and whether they were complete or incomplete.

Results. – Table 9.1.1 summarizes the frequencies of the different types of chromatid aberrations seen in the aberrant cells at the different sampling times. The table also gives the proportion of incompleteness of the different aberration types at each sampling time. It should be noted that these sampling times represent progressively longer times from metaphase and, therefore, give approximate indication of G_2 and S cells.

Several results are of interest in Table 9.1.1. First, the frequency of aberrant cells resulting from terminal deletions decreases the farther one samples from metaphase. Second, the frequency of incomplete interchanges increases as the cells approach mitosis. Third, the ratio of terminal deletions to incomplete isochromatid deletions goes from 6:1 to about 1:1.

Another feature of these data is given in Table 9.1.2. The ratio of symmetrical to asymmetrical interchanges increases as the cells approach mitosis.

Discussion. – The data on the ratio of terminal deletions to incomplete isochromatid deletions indicate that Revell obtained his expected 2.5:1 ratio fortuitously. Revell sampled his cells 6 hr after irradiation; the present 6-hr sample shows a ratio of 3.4:1, a value not drastically different from 2.5:1. The data indicate one of two things: either terminal deletions and isochromatid deletions are not the results of similar complete or incomplete intrachanges, or the probability of getting one type of intrachange vs the other is not equal.

The changing ratio of symmetrical to asymmetrical interchange indicates that the geometrical relationships of the chromosomes in the interphase nucleus that determine the type of interchange that will be formed change as the cell approaches mitosis. Further experimentation on this problem

Table 9.1.1. Frequency of the Various Types and Incompleteness of Chromatid Aberrations at Different Times of the Cell Cycle

Time Interval (hr)	Total Abnormal Cells Scored	Terminal Deletions (%)	Isochromatid Deletions (%)	Chromatid Interchanges (%)	Isochromatid Deletions Incomplete (%)	Chromatid Interchanges Incomplete (%)	Ratio Terminal vs Incomplete Isochromatid Deletions
2	309	30.1 ± 3.1	7.1 ± 1.5	63.1 ± 4.5	68.2 ± 17.7	23.1 ± 3.4	6.1:1.0
4	300	38.7 ± 3.5	37.0 ± 3.5	45.0 ± 3.9	18.9 ± 4.1	11.1 ± 2.9	5.5:1.0
6	300	24.7 ± 2.9	50.7 ± 4.1	46.3 ± 3.9	14.5 ± 3.1	6.5 ± 2.2	3.4:1.0
8	265	15.8 ± 2.4	67.9 ± 5.1	34.0 ± 3.6	14.4 ± 2.8	7.8 ± 2.9	1.6:1.0
10	300	18.3 ± 2.5	59.0 ± 4.4	29.3 ± 3.1	31.6 ± 4.2	4.5 ± 2.3	1.0:1.0
12	283	12.4 ± 2.1	49.5 ± 4.2	40.0 ± 3.8	27.7 ± 4.4	2.6 ± 1.5	0.9:1.0
Total	1759	18.9 ± 1.0	44.5 ± 1.6	45.3 ± 1.6	22.3 ± 1.7	10.4 ± 1.1	1.9:1.0

Table 9.1.2. Frequency and Ratio of the Two Types of Interchange at Different Times of the Cell Cycle

Time Interval (hr)	Symmetrical Interchanges (%)	Asymmetrical Interchanges (%)	Triradial Interchanges (%)	Ratio Symmetrical vs Asymmetrical Interchanges
2	34.6 ± 3.3	28.5 ± 3.0	1.9 ± 0.8	1.25:1.0
4	21.0 ± 2.7	24.0 ± 2.8	10.0 ± 1.8	0.88:1.0
6	21.3 ± 2.7	25.0 ± 2.9	5.7 ± 1.4	0.85:1.0
8	13.6 ± 2.3	20.4 ± 2.8	6.0 ± 1.5	0.67:1.0
10	11.3 ± 1.9	18.0 ± 2.4	8.7 ± 1.7	0.63:1.0
12	14.7 ± 2.3	25.3 ± 3.0	12.6 ± 2.1	0.58:1.0

is necessary, and the experiments are in progress at this time.

References

[1]S. H. Revell, *Mutation Res.* **3**, 34–53 (1965).

9.2 METRIC ANALYSIS OF THE HUMAN KARYOTYPE

M. A Bender M. A. Kastenbaum[1]
J. B. Davidson[2]

Introduction. – Although the human karyotype has been studied extensively in recent years, surprisingly little effort has been devoted to metric analysis of the normal human chromosome set. Two parameters, the relative lengths of the two chromosome arms (or some permutation such as total length and arm ratio), are used by the cytogeneticist in order to distinguish the various chromosomes in a set. At present this is almost universally done by visual comparison during the production of the traditional "cut and paste" karyotype from an enlarged photograph of a chromosome spread. This practice is unsatisfactory both because of the time consumed and because of its subjective nature.

Detailed metric analysis of the human chromosome set would be of value in several ways. First, judgments about which chromosomes were which, and whether or not it is possible to distinguish various chromosome pairs from each other, could be made objectively. Second, statistical analysis of the chromosome measurements from a large sample of the human population would be possible, thus establishing exactly what constitutes a "normal karyotype." Third, such a statistical statement of what constitutes the normal human chromosome set would permit the partial, or perhaps even the complete automation of the process of chromosome analysis. Large digital computers would be used, thus providing great speed and accuracy.

Methods. – In order to provide the measurements necessary for the development of methods for the statistical analysis of chromosome arm lengths, a series of cells was selected from one peripheral leukocyte culture of a normal male. The same selection criteria, including lack of chromosome overlap and correct chromosome number, were used for selecting cells for conventional karyotype analysis. A set of photographic enlargements was then made of each of the selected cells, and each chromosome in each cell was assigned a code number. Independently, an inexperienced individual measured the length of each chromatid of each arm of each chromosome in the cell, while an experienced cytogeneticist prepared a conventional karyotype. The chromosome pairs assigned by the cytogeneticist were then used to "decode" the arm measurement list.

To determine the measurement variability and the "interoperator" variability, several cells were remeasured at various times by the same individual and by a series of three other individuals.

A simple measuring aid was constructed to speed up the measurement process and make it more accurate. It consisted simply of a digital voltmeter which reads a voltage proportional to the distance between the points of a pair of dividers. A linear motion potentiometer was attached to one pair of points of a pair of proportional dividers and connected as a voltage across a constant voltage source. When a foot switch is pressed, a digital pointer records the voltage value corresponding to the divider point separation. With this device the operator measures one cell in about 4 min. Formerly, without the device, this job took 30 min and was a good deal less accurate.

For the purpose of the statistical analysis the identifications of individual chromosomes by the cytogeneticist are assumed to be absolutely correct. The actual measured arm lengths are normalized to percentages of the total lengths of all the chromosomes within each cell. A bivariate vector of means is then computed, and simultaneous two-sided confidence regions are estimated for each chromosome pair. These regions take the form of ellipses in the plane of the long arm and short arm means, with the center at the point specified by the mean lengths of the long and short arms.

Results and Discussion. – The repeated measurements made on the same cell by the same operator showed very little variation. The measurements of the same cell by different individuals were much more variable. This is apparently caused by differences in what different operators assume to be the extreme ends of the

chromosome. Neither the measurement nor the interoperator variation, however, was as great as the variation between the different cells in the series as measured by the same operator.

Figure 9.2.1 shows a Calcomp plot of the mean arm measurements from a group of seven of the cells with their 95% confidence regions. Our analysis is far from complete, and the plot is shown only to illustrate one approach to the problem. It is interesting, however, that the failure of the confidence regions to overlap for some individual chromosomes within what are usually considered to be indistinguishable groups of pairs (chromosome 16 in the "E" group, for example) suggests that they may actually be individually identifiable by means of arm length measurements.

References

[1]Mathematics Division.

[2]Instrumentation and Controls Division.

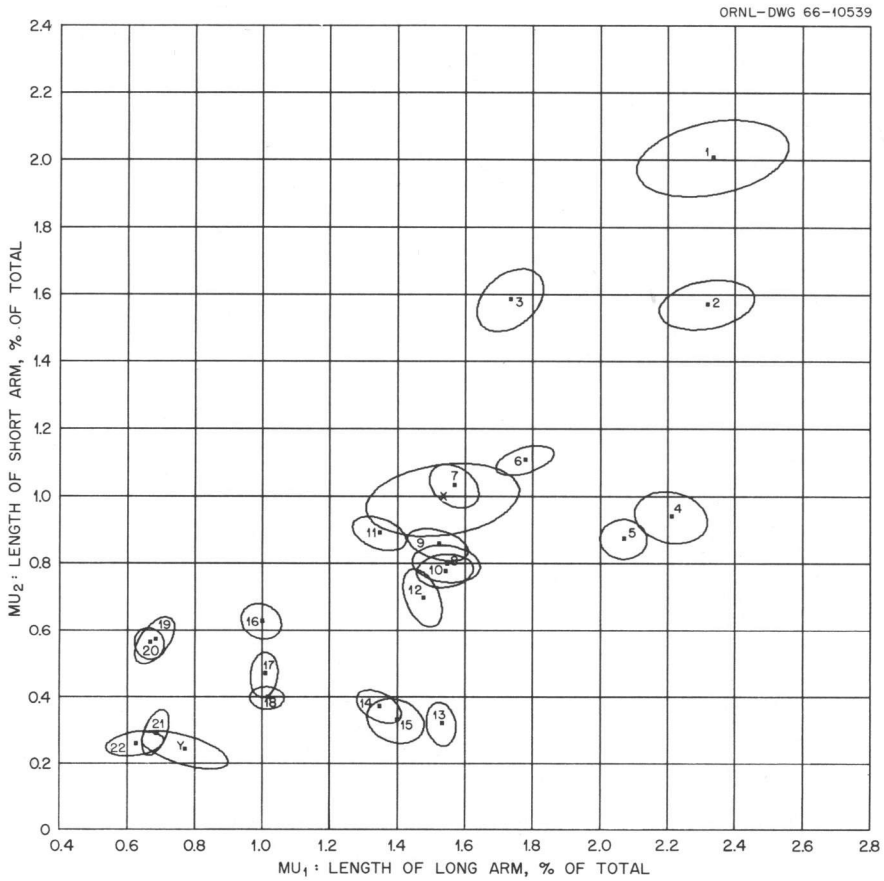

Fig. 9.2.1. Calcomp Plot of Mean Chromosome Arm Lengths, Together with Their 95% Confidence Regions, from a Series of Seven Cells from One Male.

Mammalian Recovery

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Secondary Disease (10.1–10.4)

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D. B. Thomas[c]
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Recovery in vitro (10.5, 10.6)

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Hemopoietic and Lymphatic Stem Cells (10.7–10.9)

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[e]Student trainee.
[f]Leave of absence to Weizmann Institute of Science, Rehovoth, Israel.
[g]Temporary.

10.1 EXPERIMENTAL DESIGN APPROACH TO CONTROL OF MORTALITY FROM SECONDARY DISEASE IN RADIATION CHIMERAS[1]

C. C Congdon

One central feature of secondary disease in radiation chimeras is the observation that spontaneous recovery from the disease occurs with persistence of the transplanted bone marrow cells. A reexamination of old mortality studies revealed that age of donor cells, day of cell injection, number of cells, and sex might be important as single or interacting factors in determining whether animals lived or died during the 90-day period after irradiation and foreign marrow therapy.

After carrying out a series of experimental designs it was found that the four factors chosen for study could be manipulated to reduce 90-day mortality in homologous bone marrow chimeras to about 5%.[2,3] Forty million three-day-old bone marrow cells of either sex given approximately one day after 950 r represented the best treatment conditions. Marked interaction between age of donor and number of cells injected was noted.

A new experimental design in which the donor marrow cells were of rat origin rather than homologous has been completed (Fig. 10.1.1). The central composite design for three factors consisted of 15 groups of 10 mice each for both sexes, making 30 groups or 300 animals. An extra male and female group were included at the center point

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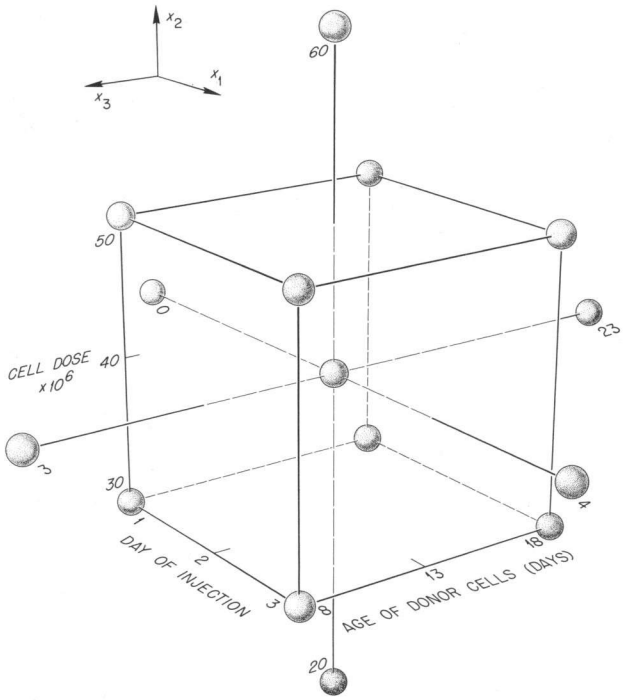

Fig. 10.1.1. Central Composite Design for Three Factors.

of the design for a total of 32 groups or 320 mice. Results of this experiment will not be available until all mice have been observed for 90 days and the survivors tested for chimerism.

This first heterologous design is intended to screen for the possibility that survival of rat-mouse bone marrow radiation chimeras might be influenced by the same factors and general levels of factors that nearly completely eliminated mortality from secondary disease in homologous bone marrow radiation chimeras.

Additional experimental designs are now being developed to determine that portion of the factor space that gives greatest mortality from secondary disease in homologous bone marrow treated mice. A more ambitious undertaking to gain insight into the pathologic, biochemical, and immunologic mechanisms that cause death in secondary disease is now being actively considered, using experimental designs with multiple end points and different time intervals for end points other than mortality.

References

[1]Work carried out in collaboration with M. A. Kastenbaum and D. A. Gardiner, Mathematics Division.

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[3]C. C Congdon, M. A. Kastenbaum, and D. A. Gardiner, submitted for publication in *Journal of the National Cancer Institute.*

10.2 ALTERATION IN RATE OF RECOVERY OF HEMOPOIETIC CELLS OF THE BONE MARROW BY INCREASED PRESSURE FOR ERYTHROID DIFFERENTIATION

A. L. Kretchmar[1]

Introduction. – If the mixed population of cells of the fixed hemopoietic sites are derived from a single primitive progenitor cell type ("stem cell"), it would seem reasonable to expect that forced differentiation from this primitive population while it was recovering from irradiation would reduce the size and rate of recovery of the population. The mammalian recovery experiment has been used to test this expectation in the following experiments. The results, while preliminary, indicate that the rate of recovery of the total population of marrow cells and of that subpopulation that can form macroscopic spleen colonies is, indeed, reduced by forced erythroid differentiation.

Methods. – Mice were lethally irradiated (950 r total-body x irradiation) and then given 20×10^6 isologous bone-marrow cells from normal donors. They were allowed various times to recover before erythropoiesis was stimulated by bleeding. Approximately $\frac{1}{3}$ of the blood volume (0.5 ml) was withdrawn from the retroorbital venous sinus of each animal on four separate occasions. The second 0.5 ml was taken three days after the first, the third four days after the second, and the fourth three days after the third. This schedule of bleeding (0.5 ml every three to four days) requires an increased rate of erythropoiesis of about 3 to 4 times if a normal red cell mass is to be maintained. The same schedule of bleeding was maintained in all groups; only the time after irradiation and bone-marrow treatment when bleeding was begun was varied.

Total nucleated cells recoverable from both femurs and tibias were measured immediately after the first bleeding (unstimulated mice) and again after the fourth bleeding (erythropoiesis stimulated for ten days). In addition, the number of colony-forming units in 10^5 of these cells was measured by the Till and McCulloch spleen assay technique. From these data the total number of colony-forming units in both femurs and tibias could be calculated.

Results. – The results are plotted in Fig. 10.2.1. It can be seen that the number of nucleated cells in this aliquot of the marrow space (two femurs and two tibias) of mice given 20×10^6 isologous marrow cells after lethal irradiation recovers rapidly and "overshoots" to levels above normal by 10 days. Somewhat elevated counts appear to persist from 15 to 45 days. In contrast, the subpopulation that can form spleen colonies recovers rapidly at first but does not reach normal numbers until 45 days after irradiation and bone-marrow treatment. At 10 and 15 days the population of colony formers is only about 80% of normal size.

When erythropoiesis was forced by bleeding and this pressure for erythroid differentiation maintained for 10 days, the rate of recovery in the marrow populations was significantly altered. The number of nucleated cells in the marrow space did not recover to above normal values and no "overshoot" was noted. The recovery of the subpopulation of marrow cells that could form spleen colonies was delayed and did not reach 80% of normal until 25 days.

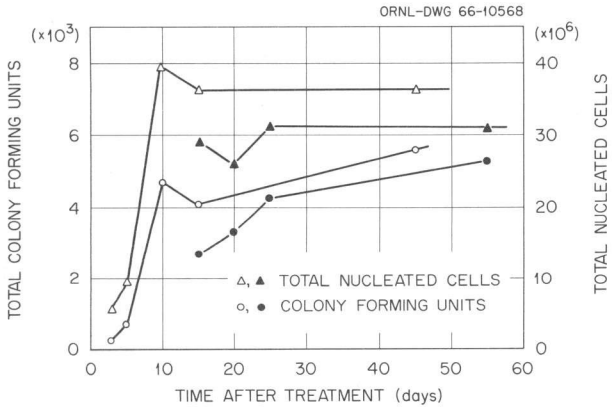

Fig. 10.2.1. Rate of Recovery of Total Nucleated Cells and Colony-Forming Units in the Marrow Space of Both Femurs and Both Tibias of Mice Given 20×10^6 Isologous Marrow Cells After 950 r Total-Body X Irradiation. The rate is shown in unstimulated animals (open symbols) and in mice with stimulated erythropoiesis (solid symbols). Control untreated mice have 32×10^6 total nucleated cells and 5.4×10^3 colony-forming units in this aliquot of the marrow space.

Discussion. – These results have important implications relative to control of size of marrow cell populations and whether differentiation occurs from a homogeneous pool of primitive progenitors or from a progenitor pool that contains subclasses of erythropoietin sensitive cells and colony-forming cells. While much further work is needed before these implications can be discussed fully, it seems important to note that the probability of a primitive cell leaving the colony-forming pool for differentiated cell lines remains less than 0.5 for at least 30 days, even when the pressure for differentiation is considerably increased. This is vital to the recovery of the animal, for the hemopoietic system can regenerate only if more than ½ of the proliferating primitive cells can escape differentiation. What mechanism protects the primitive marrow population from the pressures that lead to differentiation?

References

[1]Medical Division, Oak Ridge Associated Universities; work carried out in collaboration with the Mammalian Recovery section.

10.3 PARASITES IN THE ALIMENTARY TRACT OF RADIATION CHIMERAS

W. H. McArthur[1]

Introduction. – Recent metabolism studies relating to problems of radiation protection in mice indicate that both isologous (IBM) and homologous (HBM) bone marrow treated animals remain in positive nitrogen balance, yet the HBM-treated mice lose weight and the IBM-treated mice gain weight.[2] Loss of body weight coupled with nitrogen retention in HBM-treated animals implies nitrogen storage in some compartment. Since parasites of the alimentary tract constitute a possible compartment for nitrogen storage, we have continued to study intestinal fauna of these mice. The present report describes an attempt to better quantify the intestinal protozoans of normal and marrow-treated mice.

Materials and Methods. – Animals used in these experiments were (101 × C_3H)F_1/Cum male or female mice 12 to 16 weeks old. Each experimental animal received 950 r x irradiation and 40 × 10^6 isologous or homologous bone marrow cells. A total of 68 mice were used as follows: 18 normal, 15 x-ray, 18 isologous, and 17 homologous bone marrow treated animals. Mice were decapitated on days 1, 4, 7, 10, 13, and 14. A smear of each cecum was made, observed under the microscope, and placed in Schaudinn's fixative and stained with hematoxylin. Iron alum (4%) was used as a mordant and a weaker solution of 2% iron alum was used for destaining. Slides stained for 1 min with half-strength hematoxylin ripened with $NaHCO_3$ gave good results and did not require destaining. Cecal content of each mouse was placed on a slip of foil paper and weighed. This known amount of cecal material was diluted with about 5 cc of Tyrode's solution and passed through coarse and fine (0.1 × 0.1 mm) wire mesh filters to remove debris. The filters were rinsed thoroughly with Tyrode's and the amount of dilution recorded. A drop of the filtrate was placed on the Neubauer counting chamber, and an average protozoan count was made from four different samples of each filtrate. The average number of protozoa per gram of feces was calculated from the expression

$$\bar{c} \times V \times \frac{2.5 \times 10^3}{1 \text{ cc}} \times \frac{1}{G},$$

where

$\bar{c}$ = the average protozoan count,

V = the final volume of feces with Tyrode's,

$\frac{2.5 \times 10^3}{1 \text{ cc}}$ = a factor to convert volume from the dimensions of the counting chamber to cc's,

G = the weight of feces.

Results and Discussion. – Our recent experiments show that normal mice are practically devoid of parasites from day 1 to 14. Mice treated with homologous or isologous bone marrow possessed a large number of parasites beginning day 1 through 13. HBM-treated animals appear to have an increase in number of protozoan parasites following day 13. IBM-treated animals tend to become normal (Table 10.3.1). Observations of living

Table 10.3.1. Intestinal Flagellates in Radiation Chimeras[a]

Mouse No.		Day 1	4	7	10	13	14
Normal	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0			0		
	4	0			0		
Average		0	0	0	0	0	0
X ray	1	0	7.83×10^6	8.86×10^5	1.16×10^5		
	2	0	1.45×10^7	4.71×10^6	0		
	3	0		1.64×10^6	0		
	4	0		4.13×10^6	0		
Average		0	1.12×10^7	2.84×10^4	2.90×10^4		
Isologous	1	0	1.30×10^6	6.09×10^6	8.82×10^4	0	0
	2	0	3.14×10^6	4.45×10^6	1.46×10^6	9.07×10^4	0
	3			2.76×10^6	1.09×10^5		
	4			2.98×10^5	0		
Average		0	2.22×10^6	3.40×10^6	4.14×10^5	4.52×10^4	0
Homologous	1	0	1.01×10^8	8.62×10^7	4.08×10^6	3.28×10^5	7.56×10^6
	2	0		1.50×10^5	1.03×10^6	6.25×10^5	1.11×10^6
	3			3.25×10^6	9.40×10^4		
	4			8.26×10^7	1.20×10^5		
Average		0	1.01×10^8	2.45×10^7	1.33×10^6	4.76×10^5	4.34×10^6

[a]All values are flagellates per gram of feces.

material in earlier experiments indicated that normal and isologous mice had more protozoan parasites than did homologous mice.

Protozoan flagellates have been identified as *Trichomonas muris, Giardia lamblia,* and *Hexamita sp.* These identifications have been made from permanent stained slides. In addition to the flagellate population, trophozoite stages of endamebae and their cysts have been observed. Quantitative measurements of flagellate fauna show a high degree of parasitic infection among the HBM animals compared to isologous and normal animals (Table 10.3.1). Isologous treated animals at day 13 lose parasites and at day 14 become defaunated.

Preliminary experiments showed an increase in helminths and nematode infections in homologous mice; such findings parallel those of Friedberg *et al.*[3] Experimental animals with heavy helminthic infections revealed a reduced number of parasitic flagellates, while normal mice were almost devoid of nematodes and cestodes.

On day 4 the homologous animals reach a peak with respect to flagellate parasites, and HBM-treated animals continue to decrease in flagellate population to day 14; they then resume an upward trend at day 14.

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10.4 THE EFFECTS OF WHOLE-BODY X IRRADIATION ON THE THYMUS

D. B. Thomas[1]

Introduction. – The extreme radiosensitivity of lymphoid tissues has been demonstrated in numerous investigations (see ref. 2). Apart from the exaggerated radiosensitivity of Peyer's patches, no obvious differences in the sensitivities of different lymphoid tissues to doses of up to 1000 r have been reported.[3] Following exposures of up to 1 kr, the severity of damage to the thymus, assessed from the weight and histology of the gland 24 hr later, is roughly proportional to the dose administered, although, paradoxically, the gland appears to be less severely damaged after exposure to higher doses of 1 to 30 kr.[3,4]

The present study of the changes which occur in the mass and in the histological organization of the mouse thymus after 950 r of whole-body x irradiation was undertaken as a preliminary to an analysis of the patterns of regeneration displayed by the thymus in irradiated mice treated with intravenous injections of various lymphomyeloid cell populations. Within 24 hr of exposure to 950 r of whole-body x irradiation a pronounced decrease occurs in the mass of the thymus, and a profound degree of atrophy is evident on histological examination. Postirradiation treatment with a single intravenous injection of 10×10^6 bone marrow cells may, however, result in the restoration of the thymic lymphocytes within 8 days and the reestablishment of the normal mass of thymic tissue after 16 days or so.[5] The demonstration of cells of donor origin in the thymus glands of animals restored by injections of bone marrow cells[6–8] has led to the belief that the injected cells are responsible for the regeneration of the thymus in these animals, with no significant contribution from the irradiated host. Such restoration of atrophic lymphoid tissue in irradiated animals from the precursor-cell population of the bone marrow is consistent with the demonstration of active lymphocytopoiesis in the bone marrow.[9] Restoration by the hematopoietic precursor-cell population of fetal liver requires elucidation, however, as the hepatic hemocytoblasts of the mouse fetus show no evidence of a lymphocytopoietic differentiation potential in situ,[10] although cells derived from the blood in the hepatic sinusoids may be capable of restoring lymphocytopoiesis.[11,12]

Methods. – The thymus has been studied in groups of five $(101 \times C_3H)F_1$ female mice, which were examined at daily intervals after exposure to 950 r of whole-body x irradiation. After being weighed the thymus was fixed in Zenker-formol together with the spleen, several lymph nodes, a Peyer's patch, and the diaphysis of the left femur. Sections were stained with hematoxylin and eosin. Animals were killed immediately before examination; those which died spontaneously were not included.

Results. – A very marked decrease in the weight of the thymus occurred within four days, and histological examination revealed a severe degree of atrophy in all 25 of the glands studied during the first five days. On the sixth day, however, three out of five glands exhibited evidence of regeneration, and on the following day this was observed in all five glands. Collections of lymphocytes which were most numerous beneath the capsule were first seen on the sixth day; these persisted, becoming more widespread, so that although on the eighth day one of the five glands was atrophic, three showed clear evidence of this regenerative pattern, and in one gland very advanced regeneration was evident. Regeneration was observed in all five glands on the ninth day, and in two of these it was very advanced. The sequence culminated with evidence of advanced regeneration in all five glands on the tenth day. In following the sequence of events further, however, advanced regeneration has been observed in only two glands on the 11th day and in none subsequently. On each of the 11th and 12th days two glands showed evidence of partial regeneration only, and an increasing proportion were atrophic until on the 13th day a severe degree of thymic atrophy was seen in each of the four animals that were still surviving. The weights of the glands exhibiting the histological features of early (or partial) regeneration occupied the same range as the atrophic glands, but there was a significant increase in the weights of the glands showing advanced regeneration (Fig. 10.4.1). The weights of the atrophic glands during the initial phase of rapid decrease from the normal range on the first three days have not been represented in this diagram. Regeneration was not observed in the other lymphoid tissues, and there was no evidence of bone marrow regeneration.

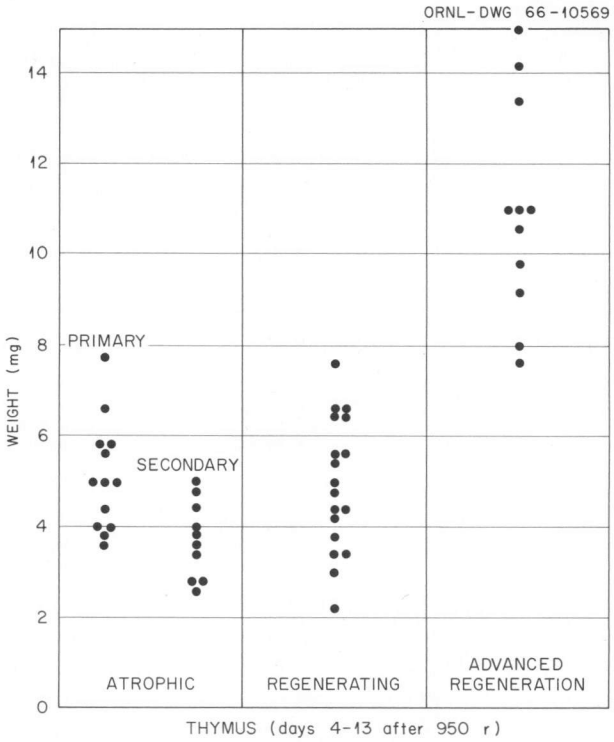

Fig. 10.4.1. Thymus Weight in Relation to Histological Appearance After 950 r. Average weight of thymus in controls is 38.5 mg; range, 31 to 46 mg.

Discussion. – After 950 r of whole-body x irradiation, regeneration commences in the thymus on the sixth day, and the regenerative process continues until on day 10 the normal organization of the thymus is restored with a partial restoration of its mass. After the tenth day, however, the effects of irradiation result in a progressive deterioration in the general condition of all survivors which culminates in death within four days. The metabolic disturbances during the period of subterminal deterioration probably affect the process of regeneration in the thymus adversely and result in secondary atrophy with decrease in weight to produce exceedingly small glands. When secondary atrophy is avoided in animals protected from the lethal effects of whole-body irradiation by the intravenous injection of lymphomyeloid cells, it is possible that regeneration of the host thymus contributes to recovery. Such a contribution may be made during the restoration of immunological reactivity in irradiated mice treated with injections of syngeneic bone marrow or fetal liver cells for which the presence of the thymus appears to be necessary.[13] A detailed reassessment of the restoration patterns of the lymphoid tissues of radiation chimeras is clearly required before the radiation chimera can be effectively exploited as an experimental model for the study of the differentiation capacities of different hematopoietic precursor-cell populations.

Conclusion. – Spontaneous regeneration has been observed in the thymus glands of mice exposed to 950 r of whole-body x irradiation, although the bone marrow, spleen, and lymph nodes remain atrophic. The thymus of the irradiated

host thus appears to be potentially capable of making a significant contribution to recovery in radiation chimeras.

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10.5 EFFECT OF RADIATION TEMPERATURE ON RADIOSENSITIVITY OF MOUSE BONE MARROW CELLS

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

Introduction. – Studies of in vitro recovery of irradiated mouse bone marrow cells are being continued.[1] To further define the variables in the methods used for these studies, we determined whether radiosensitivity of the marrow cells is a function of temperature during exposure to x rays.

Methods. – Suspensions of bone marrow cells were placed in thin-walled aluminum cups which were immersed in water maintained at 2, 25, or

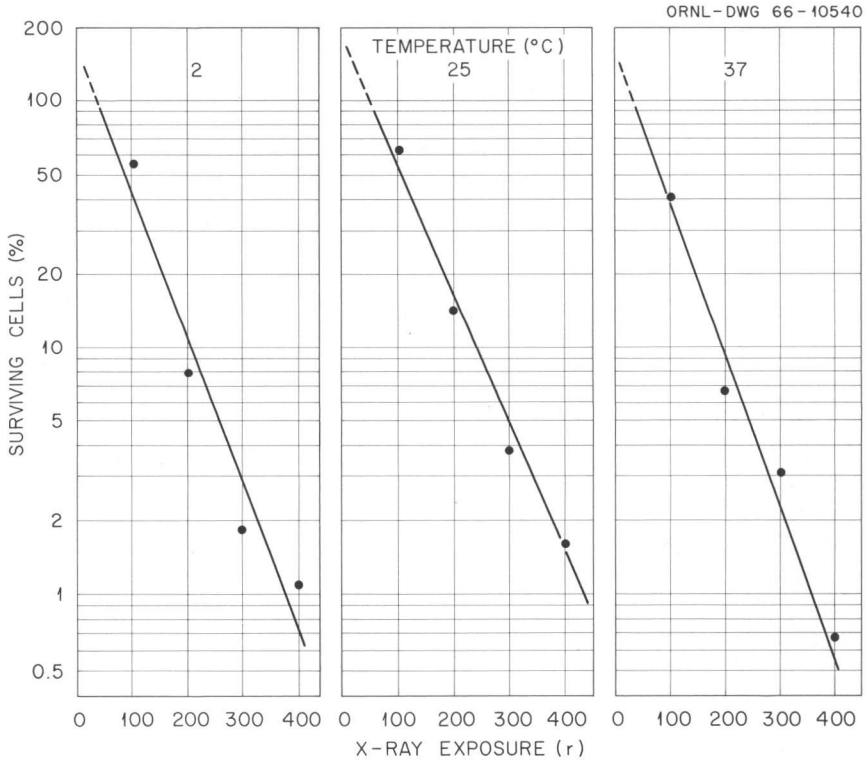

Fig. 10.5.1. Surviving Cells as a Function of Radiation Temperature.

37°C. Following irradiation at one of these temperatures, the erythropoietic capacity of the marrow cells was measured as described previously.[2]

Results. – Survival curves which are presented in Fig. 10.5.1 show that neither the D_{37} nor the extrapolation number is affected by radiation temperature. Respective D_{37}'s and extrapolation numbers at 2, 25, and 37°C are 74 r and 1.50, 80 r and 1.75, and 75 r and 1.54. Based on these measures, radiosensitivity is not dependent on temperature during exposure of mouse bone marrow cells in vitro.

Discussion. – Our data would imply that the degree of radiation injury to marrow cells is not reduced when the cells are exposed to low temperature (2°) during irradiation. The same conclusion was reached by Weiss,[3] who used HeLa cells in culture. Belli,[4] on the other hand, found that HeLa cells were more radioresistant when irradiated at 5°C than when irradiated at 40°C.

Assuming that a valid extrapolation of our data can be made to in vivo conditions, we may conclude that the radioprotection observed by rendering the whole animal hypothermic is the result of local tissue anoxia rather than a direct temperature effect. This conclusion is supported by the observation that O_2 tension is low in tissues of hypothermic (1°C) mice.[5]

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10.6 EFFECTS OF HIGHLY POLYMERIZED HETEROLOGOUS DNA ON 30-DAY SURVIVAL OF LETHALLY IRRADIATED MICE

B. S. Schnall[1]

Introduction. – It has been reported that nucleic acids given after whole-body irradiation promote recovery in rats or mice in terms of testicular damage and fertility,[2] hematopoietic tissue regeneration,[3] increased adrenal gland size,[4] and 30-day survival.[5–7] In a recent report it was found that commercially prepared DNA has a therapeutic effect on 30-day survival in rats with recovery achieved in a ratio directly proportional to the molecular weight of the DNA used.[8] Since the commercial DNA preparations used by these investigators were also available to us, we thought it of interest to test them on lethally irradiated mice.

Materials and Methods. – $(C_3H \times C_{57}BL)F_1$ male mice, three months old, were irradiated in groups of ten with exposures of 750 r (160 r/min), 825 r (160 r/min), or 850 r (25 r/min). Twenty-four hours after irradiation the mice were injected with one of three DNA preparations. The preparations used were: highly polymerized calf thymus DNA (DNA I), highly polymerized sodium salt calf thymus DNA (DNA II), and highly polymerized salmon sperm DNA (DNA III). DNA I was purchased from Mann Research Laboratories; DNA II and III were purchased from Sigma Chemical Company. All DNA solutions were prepared by stirring gently in 0.9% NaCl at 4°C for approximately 24 hr. Each experimental animal received intraperitoneally 1.0 ml containing the desired amount of DNA. Irradiated control mice were injected intraperitoneally with 1.0 ml of saline.

Results. – Table 10.6.1 shows the effects of 0.06 mg or 0.6 mg of the three DNA preparations on 30-day survival. The values in general show no indication that these nucleic acids altered the mortality pattern with the possible exception that in the mice exposed to 825 r and given 0.6 mg of DNA III, survival was slightly improved. In all other cases no apparent differences were observed between the 0.06 and 0.6 mg DNA dose levels.

Discussion and Conclusion. – Isologous DNA has been used successfully as a recovery agent in rats,[5,7] but when the same workers attempted to use heterologous (rabbit) DNA in the lethally irradiated animals, they failed to record even one 30-day survival. The successful work reported with rats on the effects of heterologous DNA on 30-day survival after lethal irradiation damage[8] does not seem to apply to mice in our laboratory.

Table 10.6.1. 30-Day Survival of Lethally Irradiated Mice Given Heterologous DNA[a]

	750 r (160 r/min)			825 r (160 r/min)			850 r (25 r/min)		
	0.06 mg	0.6 mg	Saline	0.06 mg	0.6 mg	Saline	0.06 mg	0.6 mg	Saline
DNA I, calf thymus	10/20	17/20	15/20	4/20	4/20	3/20	0/20	2/20	0/19
DNA II, calf thymus	12/20	10/20	9/18	3/20	6/20	4/15	1/20	0/20	0/23
DNA III, salmon sperm	12/18	15/18	15/16	5/20	9/18	3/18	0/20	0/20	0/16

[a]Number of mice alive/initial number.

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10.7 MODIFICATION OF POOR-GROWTH PHENOMENON

Joan W. Goodman H. B. Wheeler[1]

Introduction. – The poor-growth phenomenon, seen when tumor or normal hemopoietic cells from certain parental strains are transplanted to F_1 hybrid mice, has been reported from various laboratories in the last eight years. A satisfactory explanation for the phenomenon remains to be formulated.

Because there are major flaws in the "immune" interpretation of poor growth (i.e., the proposal offered by some investigators that parental antigens are not expressed in F_1 hybrids), we decided to look at other environmental factors, aside from those that are immunological in the traditional sense.

Experimental. – C57BL/6 Cum and (C57BL/6 Cum ♀ × DBA/2 Cum ♂)F_1 hybrid mice were used. They were exposed to 900 r total body irradiation 18 to 22 hr before receiving an infusion of 10^6 bone marrow cells. Iron-59 uptake into red cells and spleen was used to assess erythropoiesis and hence success of transplanted marrow. Some mice were pretreated with either a spleen-slice graft (placed on cut kidney surface) or an intraperitoneal injection of a cell suspension some days prior to x-ray treatment. A brief summary of results appears in Table 10.7.1. It can be seen that a parental spleen-slice graft present in the hybrid 11 to 13 days before irradiation significantly improved the subsequent growth of parental marrow. However, the stroma of the spleen graft could not have been involved, because (1) mice that had their grafts removed *prior* to irradiation and marrow transplantation also showed the effect, and (2) suspensions of spleen cells were as effective as spleen-slice grafts.

Further investigations revealed that lymph node cells, but not bone marrow or thymus (at least at the dose of 3×10^7 cells), were effective in augmenting the growth of parental marrow in F_1 hybrids (Table 10.7.2).

The mechanism of this augmentation is currently under investigation. Two possibilities come readily to mind: (1) the parental (P) lymphoid elements, in engaging in graft-vs-host (GVH) activity, proliferate and persist, forming a "proper-home" environment of P cells in which subsequently transplanted P marrow can readily clone; and (2) in taking part in G-V-H, P lymphoid cells mask with antibody F_1 cell surfaces that are ordinarily either cytotoxic or inhibitory to transplanted P marrow cells.

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Table 10.7.1. Erythropoiesis in $B6D2F_1$ Mice, Irradiated and Given B6 Marrow

Preirradiation Treatment	Days Between Treatment and X Rays	Number of Mice	24-hr ^{59}Fe Uptake by	
			Erythrocytes	Spleen
$B6D2F_1$ spleen-slice graft	12	10	1.41 ± 0.14	0.60 ± 0.08
B6 spleen-slice graft	11	10	9.68 ± 1.35	7.90 ± 1.00
B6 spleen-slice graft[a]	13	10	12.00 ± 2.57	11.58 ± 1.56
2×10^7 $B6D2F_1$ spleen cells (suspended) i.p.	14	10	0.65 ± 0.26	0.38 ± 0.08
2×10^7 B6 spleen cells (suspended) i.p.	14	10	13.24 ± 1.66	11.84 ± 1.03

[a]Graft removed one day before x ray.

Table 10.7.2. Effect of Pretreatment with Suspended Parental Cells from Different Tissues[a]

3×10^7 Parental Cells from	Number of Mice	24-hr ^{59}Fe Uptake by	
		Erythrocytes	Spleens
Spleen	25	13.40 ± 1.95	7.63 ± 0.81
Thymus	25	1.21 ± 0.11	0.67 ± 0.08
Bone marrow	22	0.47 ± 0.06	0.30 ± 0.02
Lymph nodes	24	15.38 ± 1.37	9.83 ± 0.66
b	25	0.49 ± 0.06	0.36 ± 0.04

[a]Recipients $B6D2F_1$, pretreated 21 days before x rays and 10^6 B6 marrow cells.
[b]Saline injected controls.

10.8 ABOLITION OF POOR GROWTH OF PARENTAL MARROW CELLS IN "OLD" RADIATION CHIMERAS

Joan W. Goodman Gayle C. Bosma

Introduction. – The radiation chimera, in this case a mouse exposed to heavy radiation and then given a transplant of foreign bone marrow, permits one to study proliferation of cells in an unusual environment. We wondered whether the proliferation of a second marrow transplant, within a day after a second x-ray dose of 900 r, delivered three to five months after the first such treatment (hence "old" chimeras), would be different from the first.

Experimental. – Mice of inbred parental (P) strains C57BL/6 Cum (B6), DBA/2 Cum (D2), C57BL Cum (B), C3H Anf Cum (C3), and their F_1 hybrids $B6D2F_1$ and $BC3F_1$ were used. Only mice (six to eight months old) having 100% donor-type red cells by agglutination and hemolysis test 90 to 150 days after first marrow transplantation were reirradiated. They then received 10^6 marrow cells from the C57BL parent, well-known to exhibit "poor growth" in these two combinations. The results are shown in Table 10.8.1. When the chimeras (all F_1 recipients) contained marrow, and presumably lymphatic tissue, of P type, a second transplant of identical P marrow did not exhibit poor growth. It should be noted that when B marrow was given to C3 → F_1 chimeras, its growth at one week was intermediate between that seen in B → F_1 and that in F_1 → F_1 chimeras. A similar relationship is seen on primary transplantations of B marrow into irradiated B, C3, and F_1 mice. Thus performance of the transplant appears to depend not so much on the genetic constitution of the entire host as on that of the hematopoietic and/or lymphopoietic tissue resident in the animal at the time of irradiation and transplantation.

Table 10.8.1. Erythropoiesis by F_1 Hybrid Chimeras[a] Reirradiated and Given 10^6 Parental BM Cells

Recipient	Original[b] BM Donor	Number of Mice	^{59}Fe Uptake[c] by	
			Erythrocytes	Spleens
$B6D2F_1$	B6	23	14.73 ± 1.83	8.76 ± 0.73
	D2	18	5.09 ± 1.24	3.32 ± 0.71
	B6	21	13.49 ± 1.61	7.41 ± 0.53
	D2	32	2.38 ± 0.54	1.80 ± 0.36
	B6	15	13.70 ± 1.58	9.00 ± 0.75
	D2	23	6.68 ± 1.40	3.86 ± 0.75
	B6	42	11.33 ± 1.01	6.27 ± 0.4
	F_1	71	2.21 ± 0.23	1.54 ± 0.13
$BC3F_1$	B	29	24.89 ± 2.27	9.66 ± 0.38
	C3	29	16.91 ± 1.72	8.22 ± 0.76
	F_1	15	3.14 ± 0.70	2.33 ± 0.40

[a]Mice surviving at least 90 days after treatment and containing red cells of original donor type only.

[b]Mice treated with 10^6 marrow cells after 900 r.

[c]Means ± standard errors. 0.5 μc ^{59}Fe as citrate given seven days after 10^6 B6 cells injected into reirradiated (900 r) chimeras. 24-hr uptake expressed as percentage injected ^{59}Fe dose.

10.9 HISTOLOGIC STUDIES ON POOR GROWTH OF PARENTAL BONE MARROW

Fay B. Martin Joan W. Goodman

We have known for many years that homologous mouse and heterologous rat bone marrow are not as efficient sources of cells for marrow transplants into lethally irradiated mice as is genetically identical mouse marrow. In these experiments histologic evidence of a residual host immune response suggests a reasonable explanation for the inefficiency of marrow transplantation. However, the poor growth exhibited by certain (but not all) parental (P) strains on transplantation to F_1 hybrids cannot be explained on an immune basis, as P cells are genetically compatible according to current theory and experimental findings. Many investigations of this problem in cell growth have been made,[1] and the present work was undertaken to determine the histologic features of the growth of isologous parental bone marrow in irradiated F_1 hybrid mice. The poor growth of parental bone marrow in F_1 hybrid mice has the added special feature that one parent's cells may grow well in the hybrid while the other parent's cells do very poorly during the initial phase of transplantation. Once the graft is established, cells from either parental source grow equally well. This is also apparently true in homologous and heterologous transplants.

Materials and Methods. – $B6D2F_1$ mice 12 to 16 weeks old were given a whole-body exposure of 900 r x irradiation. On day 1 after irradiation the mice were divided into four groups. One group was given B6 bone marrow, the second D2 bone marrow, the third F_1 hybrid marrow, and the fourth group served as x-ray controls; unirradiated controls were also included.

Experiment 1: The bone marrow dose was 10×10^6 cells/mouse for all three transplant groups. Five animals from each group were sacrificed on days 2, 3, 4, 5, 6, 8, 10, 17, 24, and 31. Five normal animals were sacrificed on alternate days of sacrifice and the x-ray controls sacrificed on days 1 to 5. Samples of bone marrow (sternum and femur), lymph nodes, spleen, Peyer's patches, and thymus were fixed in Zenker's fixative for histology, and the sections were stained with hematoxylin and eosin.

Experiment 2: The bone marrow dose was 1×10^6 cells/mouse for the three groups. Five animals from each group, three from the x-ray control group, and two from the normal group were sacrificed on days 2–11. Tissues were taken for histology.

Experiment 3: From previous data relating cell dose to amount of erythropoiesis, the following "equivalent" cell doses for B6, D2, and F_1 marrow were calculated and tested: B6 – 21.18×10^6; D2 – 1.34×10^6; and F_1 – 1×10^6. Five animals from each group, three from the x-ray control group, and two from the normal group were sacrificed on days 2 to 11. Tissue samples were taken for histology.

Results and Discussion. – Results of the histologic study on spleen red pulp in the first experiment showed delayed growth of transplanted cells from B6 donors into irradiated F_1 hybrid recipients, whereas D2 and F_1 cells grew faster and at nearly the same rate in the F_1 animal. With a dose of 10×10^6 F_1 or D2 cells large hematopoietic foci were regularly present in the spleen red pulp by day 4, and hyperplasia reached by day 6. For B6 donors there was about a two-day delay in achieving the same stages of transplant growth.

In experiment 2, F_1 recipients of 10^6 B6 cells did not show hyperplasia of the spleen red pulp during the 11-day period of study. Some of the recipients of D2 and F_1 marrow had hyperplastic transplants by day 7, and all were hyperplastic at the later intervals.

The third experiment was based on prior erythropoietic transplant studies with spleen uptake of ^{59}Fe where, with equal low cell doses, B6 donors give less initial uptake than D2 or F_1 donors. Iron uptake with B6 donors could be made equivalent to that obtained with the other two groups by increasing the B6 cell dose. When this was done and histologic results determined, it was noted that all three sources of transplanted cells gave equal rates of growth in the spleen red pulp.

There was no observable histologic explanation for the initial poor growth of B6 cells in the F_1 hybrid in these experiments. There was no evidence of an immune reaction in the spleen white pulp during the time when the poor-growth phenomenon was observed.

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10.10 STUDIES ON A HIGHLY LETHAL ENTERIC DISEASE OF COTTONTAIL RABBITS

C. B. Richter M. L. Simmons
J. A. Franklin

Introduction. – During the past three years Kansas cottontail rabbits (*Sylvilagus floridanus*), purchased for use in Shope papilloma studies, have suffered high mortality while held at the Receiving and Quarantine Station of this Laboratory. Mortality rates have exceeded 50%, with death almost invariably due to gastroenteritis characterized by sudden onset and brief clinical duration. Rabbits may appear normal in the afternoon or evening and be dead by the following morning. Appetite and water consumption remain normal up until the time of clinical onset. Peak mortality occurs at day 5 of confinement at R and Q and thereafter tapers off (Fig. 10.10.1). Mortality after day 20 is limited and widely scattered. Empirical methods directed at control of the problem have met with little success. With this information it was decided to attempt to establish the nature of the disease.

Methods and Results. – Post mortem observations: Typically the carcass is markedly dehydrated and the perianal fur is smeared with fecal matter. There are punctate areas of focal necrosis of the gastric mucosa with subtending submucosal

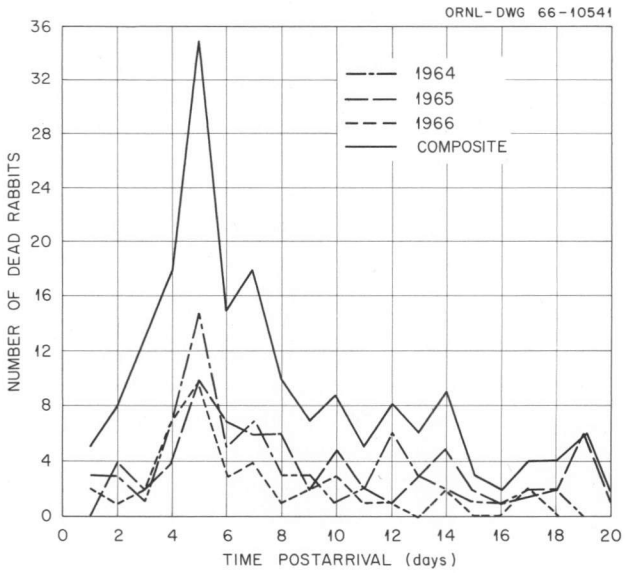

Fig. 10.10.1. Accumulated Daily Pattern of Mortality in Kansas Cottontail Rabbits with Acute Gastroenteritis (1964–66).

edema. The contents of the small intestine are liquid and often frothy. The cecum is distended by gas and foul-smelling brownish liquefied material. Usually the cecal serosa and mucosa bear streaks of hemorrhage. Excessive amounts of mucus are found in the colonic lumen, and occasionally this organ bears petechial hemorrhages. Microscopically there is cellular infiltration of the cecal and colonic mucosa. The spleen is usually atonic and atrophic. The germinal centers of the spleen, appendix, and sacculus rotundus invariably contain necrotic cells, occasionally to a marked degree. Findings in other tissues are limited.

A bacteriological study was made of 12 rabbits which were divided into three classes, for example, normal (sacrifices), clinically sick, and dead of disease. Multiple samples were taken from the viscera and different levels of the gastrointestinal tract and cultured aerobically and anaerobically on a variety of enrichment and differential media. These failed to yield any unusual or commonly accepted enteric pathogens. Coliforms were the only bacteria invariably isolated from the gastrointestinal tract, and visceral cultures were usually negative. Since it seemed possible that a known pathogenic coliform or at least a single strain of this organism might be common to diseased rabbits, cultures from six were sent to a reference laboratory for complete typing. This hypothesis was not borne out by the results reported. At least one other investigator[1] has proposed that pathogenic coliforms and/or their endotoxins may be involved in acute enteric disease of rabbits, but the results are not convincing. From our bacteriology studies, we conclude that (1) bacteremia does not occur in sick rabbits, (2) commonly accepted enteric pathogens are not the cause of the disease, and (3) a known pathogenic coliform or a single strain of coliform is probably not involved.

As yet little work has been done on transmission of this enteric disease. Attempts to infect weanling mice with cell-free filtrates and antibiotic-treated tissue emulsions have not been successful. Studies employing suckling mice and domestic rabbits are in progress. Tissue culture virus isolation techniques are proposed.

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Pathology and Physiology

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

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Physiological Effects of Radiation and Chemicals (11.4–11.6)

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Gnotobiotics (11.7)

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Epidemiology and Microbiology (11.10, 11.11)

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Participation in Cooperative Programs: AEC-NIH Chemical Carcinogenesis (22.6, 22.7)

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11.1 CONTINUING MAMMALIAN STUDIES WITH 60-Mev PROTONS IN THE OAK RIDGE ISOCHRONOUS CYCLOTRON

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N. K. Clapp K. W. Christenberry
M. C. Jernigan J. W. Conklin

Introduction. – As part of our study of the RBE of high-energy charged particles for late somatic effects in mice, we previously reported results of preliminary observations on opacification of the lens in RF/Up female mice up to 200 days following exposure of the eyes to 35- and 57-Mev protons in the ORIC.[2] From these results we tentatively concluded that 35- and 57-Mev protons have essentially the same effectiveness as 300-kvp x rays for induction of lens opacities. Results of observations on a few mice now out to 500 days have not affected these conclusions.

Results and Discussion. – The dosimetric studies and irradiation procedures worked out for whole-body exposure of mice in a broad beam of 57-Mev protons have been described.[3] The mice are exposed four at a time, broadside to the beam, in 1-in.-diam rotating tubes. The maximum proton energy available in the ORIC now is 60 instead of 57 Mev, further reducing the possibility that the region of the mouse body on the exit side of the beam will receive a component of high-LET radiation as a result of particle straggling. About 1000 eight-week-old RF/Up females (approximately two-thirds of the total to be treated) have been given whole-body irradiation with 57- to 60-Mev protons for the study of delayed somatic effects, with a comparable group being set up simultaneously as x-irradiated controls (300 kvp). The mice are subjected to a single exposure of 0 (sham irradiated), 50, 100, 200, 300, or 400 rads of protons or x rays, except for smaller numbers at higher dose levels to obtain preliminary estimates of the LD 50/30. About half of the animals are weighed individually at frequent intervals, beginning early in the postirradiation period, and a large number in all dose groups are examined periodically with the slit lamp to follow development of lens opacities. Checks for dead and moribund mice are made in all cages daily. More frequent checks are made of cages with sick mice to ensure satisfactory specimens for microscopic examination. Necropsies routinely include the cranial contents.

The only results on delayed effects from whole-body 60-Mev proton exposures to date are the preliminary data on lens opacification and body-weight changes from the first groups irradiated. The lens data (to 150 days after treatment) are in essential agreement with the earlier findings on 35- and 57-Mev protons mentioned above. Mouse weights following the acute postirradiation period have increased steadily to date (150 days after exposure) in all treatment groups, with no appreciable radiation-induced decrease in weight gain being evident in either proton- or x-irradiated series at sublethal dose levels (Figs. 11.1.1 and 11.1.2). The preliminary estimates of the LD

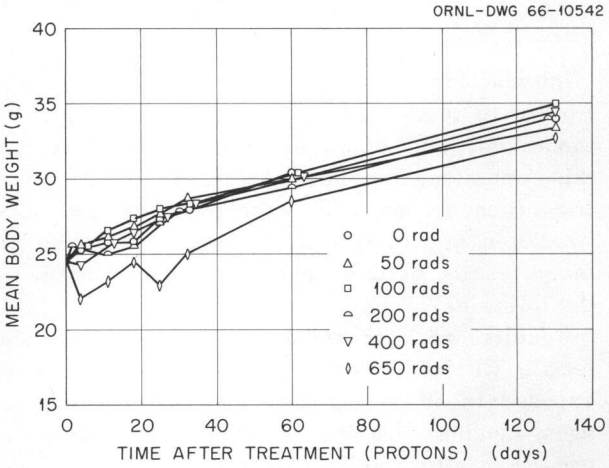

Fig. 11.1.1. Mean Body Weights of Mice After Irradiation with 60-Mev Protons. Mean body weight was 24.5 g at day 0.

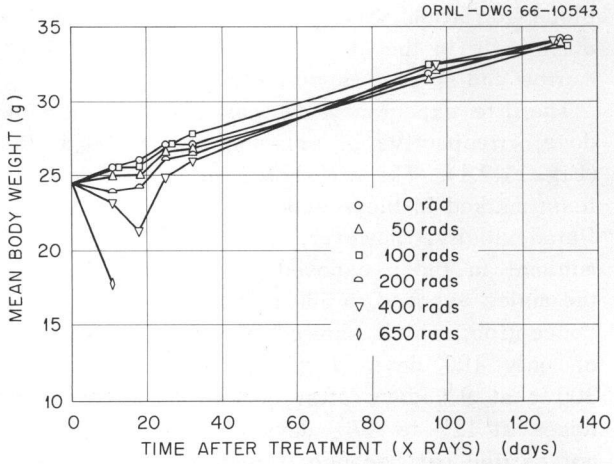

Fig. 11.1.2. Mean Body Weights of Mice After Irradiation with 300-kev X Rays. Mean body weight was 24.5 g at day 0.

50/30 are 600 and 525 rads for protons and x rays respectively.

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[1]Electronuclear Division.

[2]E. B. Darden, Jr., *et al.*, *ORNL Space Biology Program Annual Report, June 30, 1966*, ORNL-TM-1217, p. 7.

[3]*Ibid.*, p. 3.

11.2 INFLUENCE OF AGE AT IRRADIATION ON SUSCEPTIBILITY TO RADIATION-INDUCED LIFE SHORTENING IN RF MICE

J. W. Conklin R. A. Popp[1]

Introduction. – The influence of age on susceptibility to radiation-induced life shortening is of theoretical and practical interest. To explore this question, we have analyzed effects of x irradiation on the life expectancy of RF mice irradiated at widely differing ages. The preliminary results of this analysis are summarized in the following.

Results and Discussion. – Noninbred male and female RF mice of the Oak Ridge subline were exposed to 50 to 400 r of whole-body x rays at ages ranging from 9½ days after conception to one year after birth. Irradiation in utero was carried out by whole-body exposure of pregnant mice at (1) 9½, (2) 12½ and 14½, and (3) 17½ days after copulation, to expose the hemopoietic system of fetuses at stages of development when blood formation was found to be localized predominantly in the (1) yolk sac, (2) liver, and (3) marrow and spleen respectively.

The life expectancy decreased with increasing dose, irrespective of sex and age at irradiation (Fig. 11.2.1). The life shortening was, in general, least marked in those exposed at one year of age. Paradoxically, however, the effects were also minimal in those exposed prenatally, except for the males exposed to 300 r at 14½ days following conception, which showed a mean survival time of only 190 days (Fig. 11.2.2). Exposure to 300 r at 9½ days after conception or to larger doses at 12½ to 17½ days after conception was not carried out because it resulted in high intrauterine lethality. None of the groups reported herein, however, showed evidence of prenatal or neonatal mortality attributable to irradiation.

The diseases observed in association with life shortening were similar to those noted earlier in mice of the same strain except in males dying within one year after exposure to 300 r at 14½ days following conception, which failed to show the spectrum of lesions typical of aging RF males. Instead, they manifested stunting of body growth, microcephaly, hyperirritability, a tendency toward ulcerative dermatitis (possibly from fighting or from mite infestation), and premature onset of intercapillary glomerulosclerosis with signs suggestive of incipient periarteritis. There was no detectable increase in the incidence of leukemia or solid tumors in this or any other group of prenatally exposed mice.

The decrease in life shortening with increasing age after weaning agrees with published observations in mice[2] and rats.[3] The reduced response of our one-year-old mice may be partly ascribed

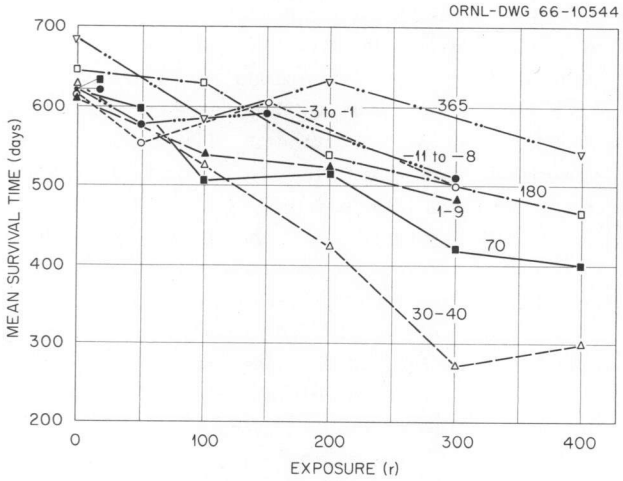

Fig. 11.2.1. Mean Survival Time of Females in Relation to Magnitude of Exposure and Age at Irradiation.

● Exposure 8–11 days before birth, i.e., 9½ to 12½ days after conception (225–250 mice per group).

○ Exposure 1–3 days before birth, i.e., 17½ to 19½ days after conception (225–300 mice per group).

▲ Exposure 1–9 days after birth (75–150 mice per group).

△ Exposure 30–40 days after birth (40–50 mice per group).

■ Exposure 70 days after birth (45–100 mice per group).

□ Exposure 180 days after birth (39–100 mice per group).

▽ Exposure 365 days after birth (37–45 mice per group).

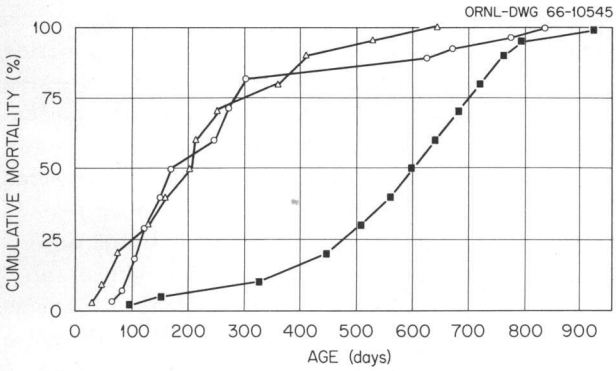

Fig. 11.2.2. Cumulative Mortality in Males Exposed to 300 r 14½ days After Conception. ○, first experiment (exposure in 1961); △, second experiment (exposure in 1963); ■, pooled controls.

to their failure to survive the long induction period for the life-shortening effects in question, but the induction period in the different age groups differed enough to suggest that susceptibility itself varied with age. Age differences in life shortening may be expected to be particularly large when the effects on survival depend on induction of a specific disease, the susceptibility to which varies markedly with age (e.g., thymic lymphoma and intercapillary glomerulosclerosis). The reduced susceptibility observed in our aging mice is, therefore, being analyzed in relation to the age distribution and incidence of radiation-induced diseases in the different age groups.

The relatively low susceptibility of fetal mice to radiation-induced life shortening, except in the case of the males exposed to 300 r at 14½ days after conception, was unexpected and cannot yet be explained. It contrasts with the apparently high susceptibility of fetal rats to radiation-induced life shortening.[4] The marked life shortening in the males exposed to 300 r at 14½ days of age was, likewise, unexpected and remains to be explained. Although it was associated with stunting of growth, microcephaly, hyperirritability, chronic ulcerative dermatitis, glomerulosclerosis, and signs suggestive of incipient polyarteritis, the possible role of these lesions in its pathogenesis is unknown. By themselves, the glomerulosclerosis and associated arteritis appeared too mild to have contributed to death. Moreover, these lesions occurred to the same extent in females, which did not show drastic life shortening, as in males.

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11.3 POLYARTERITIS IN AGING RF MICE

J. W. Conklin W. D. Gude

Introduction. – In contrast to its high incidence in elderly human beings, cardiovascular disease has not been reported heretofore as a regular accompaniment of senescence in the mouse. It is noteworthy, therefore, that histologic examination of the heart in aging RF mice has disclosed polyarteritis of the aorta and its branches with unexpectedly high frequency.

Results and Discussion. – The lesion is characterized in its acute stages by infiltration of polymorphonuclear and mononuclear cells throughout the wall of the affected vessel, with fibrinoid necrosis of the media. Aneurismal dilatation, rupture, and fibrosis of the vessel have been noted in the later stages. Sites of predilection include the proximal aorta and the coronary, renal, uterine, ovarian, and splenic arteries. The incidence and age distribution of the lesions are not known precisely, but the disease has been observed in up to 20% of the mice older than nine months that have been examined histologically in various experiments. It has not been noted in younger mice, nor has its incidence been detectably affected by whole-body irradiation early in life. The pathogenesis of the disease is under study.

11.4 LIFE CYCLE OF MEGAKARYOCYTES

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

Introduction. – We previously postulated that the nuclei of megakaryocytes become polyploid during a period of active DNA synthesis prior to differentiation or maturation of the cytoplasm. To explore this possibility we have injected rats with tritiated thymidine (^{3}HTdr), measured the amount of DNA in individual megakaryocytes of

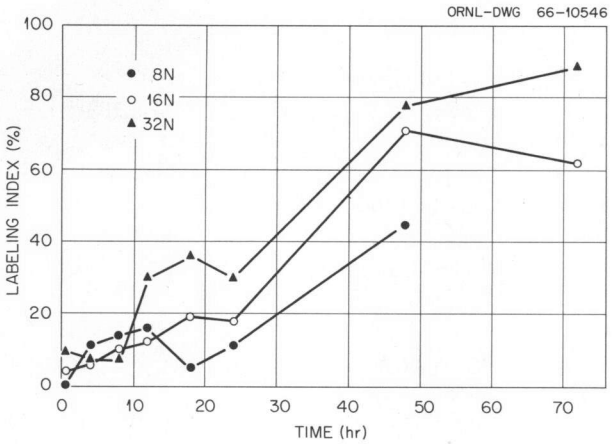

Fig. 11.4.1. Labeling Index of Megakaryocytes of 8-, 16-, and 32-*N* ploidies.

these rats by microspectrophotometric determination of light transmittance through the nuclei of Feulgen-stained cells, and subsequently made autoradiograms of the same cells to determine the labeling index. The rats were killed at intervals from 30 min to 72 hr after injection of ^{3}HTdr, and bone marrow smears were made. Individual megakaryocytes were placed in ploidy classes according to the amount of DNA in their nuclei, and the percentage of the measured cells in each ploidy class that was labeled at intervals after injection of ^{3}HTdr was determined.

Results and Discussion. – The labeling index was similar in the 8-, 16-, and 32-*N* ploidy classes, being a few percent in each class soon after ^{3}HTdr injection and increasing to 60 to 90% at 72 hr (Fig. 11.4.1). Thus some cells in all ploidy classes utilized ^{3}HTdr in the synthesis of DNA. Other studies have indicated that the megakaryocytes that take up ^{3}HTdr immediately after its injection are young, by morphological criteria, particularly the characteristics of the cytoplasm. Since the initially labeled megakaryocytes included cells of all ploidy values, the hypothesis that megakaryocytes go through a period of nuclear replication prior to maturation of the cytoplasm is supported by the results of this experiment.

11.5 VIABILITY OF IRC 741 LEUKEMIA CELLS AFTER STORAGE IN LIQUID NITROGEN

D. M. Morris V. K. Jenkins

Introduction. – In the hope of obviating the necessity to make frequent serial passages of the Dunning IRC 741 lymphoma and of providing a stable tumor material, we have initiated a series of experiments to study the effect of freezing and storage of this material. It has been demonstrated that the viability and biological characteristics of a variety of tumors,[1] including leukemias and lymphomas,[2] may be preserved for long periods after storage in the frozen state. Upton *et al.*,[3] using the method described by Albright *et al.*,[4] were able to preserve the viability of mouse myeloid leukemia cells. We have applied the same methods to the preservation of IRC 741 leukemic tumor cells. We report here that this tumor can indeed be stored in liquid nitrogen for several months with little or no loss of potency as measured by the ability of relatively large numbers of cells to induce leukemia in Fischer rats.

Results and Discussion. – A cell suspension was prepared by mincing a subcutaneous tumor from a Fischer 344 rat with surgical scissors in cold sterile Tyrode solution. The suspension was then passed through wire gauze to remove the large particles and allowed to sediment in the cold for 30 min. The relatively detritus-free material was drawn off the top with a 26-gage needle. A cell count was made with a hemocytometer using the method of Schrek,[5] and a final concentration of 44,000 viable (eosin negative) cells/mm^3 was prepared in Tyrode's solution containing 10% dimethyl sulfoxide. Aseptic technics were used throughout the experiment, and all procedures were done on ice. One-milliliter aliquots were placed in glass vials, sealed, and frozen at the rate of 1°C/min in the Linde BF 3-1 liquid-nitrogen freezer to a temperature of –50°C. Adjustment was made so as to pass through the heat of fusion temperature as rapidly as possible. The vials were then stored in liquid nitrogen for 161 days. At the end of this period three vials were thawed in a water bath at 37°C for 1.5 to 2 min and then held at ice temperature until inoculation. Cell counts were made, and several rats were injected subcutaneously with 0.1 ml of the suspension, approximately 2.5×10^6 cells (Table 11.5.1).

A tumor developed at the site of injection in every rat, and they all subsequently died of leukemia. The mortality rate, incidence, latency, and survival time of rats receiving frozen and thawed tumor cells were comparable to those of rats receiving similar numbers of fresh tumor cells. There was no significant difference in

Table 11.5.1. Response of Fischer Rats to Subcutaneous Inoculation of IRC 741 Leukemic Cells After Storage in Liquid Nitrogen

Treatment of Tumor Cells	Number of Viable Cells per 0.1 ml Injection	Number of Rats Injected	Mean Latency (days)	Mean Survival Time (days)	Tumor Size at Death (cm^3)
Frozen (vial 1)	2.3×10^6	6	6.57	13.5	20.5
Frozen (vial 2)	2.9×10^6	6	7.00	13.8	21.6
Frozen (vial 3)	2.6×10^6	10	7.25	14.6	17.5
Fresh	13×10^6	8	7.0	14.2	
Fresh	13×10^5	8	9.7	16.9	
Fresh	13×10^5	10	5.5	12.7	

the preparations in the three vials. Further experiments are in progress to determine whether slight changes in potency due to freezing and thawing may occur which were not detectable under the conditions of the present experiment.

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11.6 ABILITY OF RAT BONE MARROW TO PRODUCE SPLEEN COLONIES IN IRRADIATED HOSTS

D. M. Morris T. T. Odell, Jr.
C. W. Jackson

Introduction. – It has been amply demonstrated that bone marrow cells transfused intravenously into an irradiated mouse establish sites of proliferation in the host's spleen, resulting in the development of large colonies of cells which can be detected grossly as well as histologically within one to two weeks. Although it has been shown that rat marrow promotes recovery from radiation damage in rats and in mice, the colony-forming capacity of rat marrow has not been reported, to our knowledge. Experiments were undertaken to determine whether rat marrow transfused into irradiated rats or mice would result in the growth of colonies in the spleen. Spleen weight and uptake of ^{59}Fe by the spleen were also examined.

Results and Discussion. – Transfusion of mouse marrow cells into irradiated syngeneic recipients produced colonies in the spleen, as expected. Marrow cells of CD rats (Sprague-Dawley derived) also produced colonies in the spleens of irradiated (900 r) (C3H $\times$ C57 B1) F_1 mice when the cell dose was 5×10^5 or greater. When rat marrow was transfused into irradiated recipient rats of the same strain, the outcome depended on the strain of rat. No gross colonies were observed in any of 58 CD rats transfused with CD marrow, although a wide range of cell doses from 1×10^5 to 1×10^9 cells were investigated and a variety of radiation exposures (900, 1000, and 1200 r) were used. On the other hand, gross colonies were seen in seven of ten Fischer rats exposed to 1000 r and transfused with 4×10^5 Fischer marrow cells. Although no gross colonies were apparent in spleens of CD rats, spleen weight and ^{59}Fe uptake were generally greater than in irradiated controls not transfused with marrow cells (Table 11.6.1). In addition, microscopic aggregates of cells were present in all spleens of marrow recipients.

It is thus apparent that the formation of grossly visible spleen colonies in rats is dependent

Table 11.6.1. Spleen Weight and ^{59}Fe Uptake in Irradiated Rats Transfused with Rat Bone Marrow Cells

Strain	Number of Cells	Radiation (r)	Time After Transfusion (days)	Index[a] of Spleen Weight	Index[a] of ^{59}Fe Uptake	Number of Rats (Exptl.)
CD → CD	1×10^6	900	8	0.9	1.5	4
CD → CD	1×10^8	900	8	1.1	2.1	4
CD → CD	1×10^8	1000	8	1.6	3.5	5
CD → CD	1×10^8	1000	13	2.1	2.5	5
CD → CD	1×10^8	1200	8	1.3	1.3	3
CD → CD	1×10^8	1200	13	2.2	6.5	3
F → F	4×10^5	1000	13	1.4	2.5	10
F → F	1×10^8	1000	13	1.8	3.0	5

[a]Index equals mean spleen weight or ^{59}Fe uptake value of each experimental rat group divided by the respective mean values of the pertinent control rats similarly treated but not transfused with marrow cells.

upon the strain of rats used. This end point cannot be used as a measure of proliferation of marrow cells after transplantation in CD rats. It is interesting to note, however, that colony formation is observed in the mouse spleen after inoculation of the mouse with CD rat bone marrow cells, thus suggesting that the lack of colony formation observed in the CD rat spleen is not due to a deficiency in proliferative ability of the rat marrow cells but rather to a less favorable environment for colony formation provided by the rat spleen.

11.7 PRELIMINARY OBSERVATIONS ON AGING IN IRRADIATED AND UNIRRADIATED GERMFREE RFM MICE

H. E. Walburg, Jr. G. E. Cosgrove

Introduction. – A previous study[1] suggests that the microbial flora and fauna need not affect aging processes drastically. This is a preliminary report of a larger experiment designed to (1) clarify further the role of the microbial environment in aging, (2) study certain diseases which seem to be influenced by the microbial environment (e.g., amyloidosis, cecal volvulus, glomerulosclerosis, and auricular thrombosis), and (3) study the effect of the microbial environment on the late somatic effects of radiation.

Results and Discussion. – Germfree and conventional RFM/Un mice exposed to 300 r of x radiation at five to six weeks of age or held as sham-irradiated controls were examined with respect to life-span and incidence of disease. Unirradiated germfree RFM mice had an increased survival rate in the middle one-third of the life-span when compared with their conventional counterparts (Fig. 11.7.1). This difference in survival appears to be associated with infectious diseases of the conventional mice and tends to diminish in the last third of life, so that survivorship is not greatly different in the last decile. In irradiated RFM mice, the germfree groups also had greater survival in the middle third of the life-span, but the differences were largely obscured by the high incidence of leukemia and could be ascribed solely to the absence of myeloid leukemia in the germfree mice. Thus, although the microbial flora and fauna may alter the form of the survival curve and may increase the mean age of death, they do not seem to increase the maximum length of life.

Although the data are incomplete, early results suggest that, as in the previous cited experiment with ICR mice, the detectable microbial environment plays little role in the spontaneous development of neoplasia, either lymphatic leukemia or solid tumors. In irradiated RFM/Un mice there is likewise no difference between germfree and conventional mice as regards the development of lymphatic leukemia (Fig. 11.7.2). On the other hand, irradiated germfree mice have failed to develop myeloid leukemia, which is a char-

acteristic radiation response of the conventional RFM mouse (Fig. 11.7.3). The absence of myeloid leukemia in germfree mice and the reduced incidence of myeloid leukemia in recent experiments (1964) using conventional mice from disease-controlled colonies, as compared with older experiments (1956) using conventional mice from uncontrolled colonies, may be due to the effect of the microbial flora on granulocytopoiesis. This possibility is being explored.

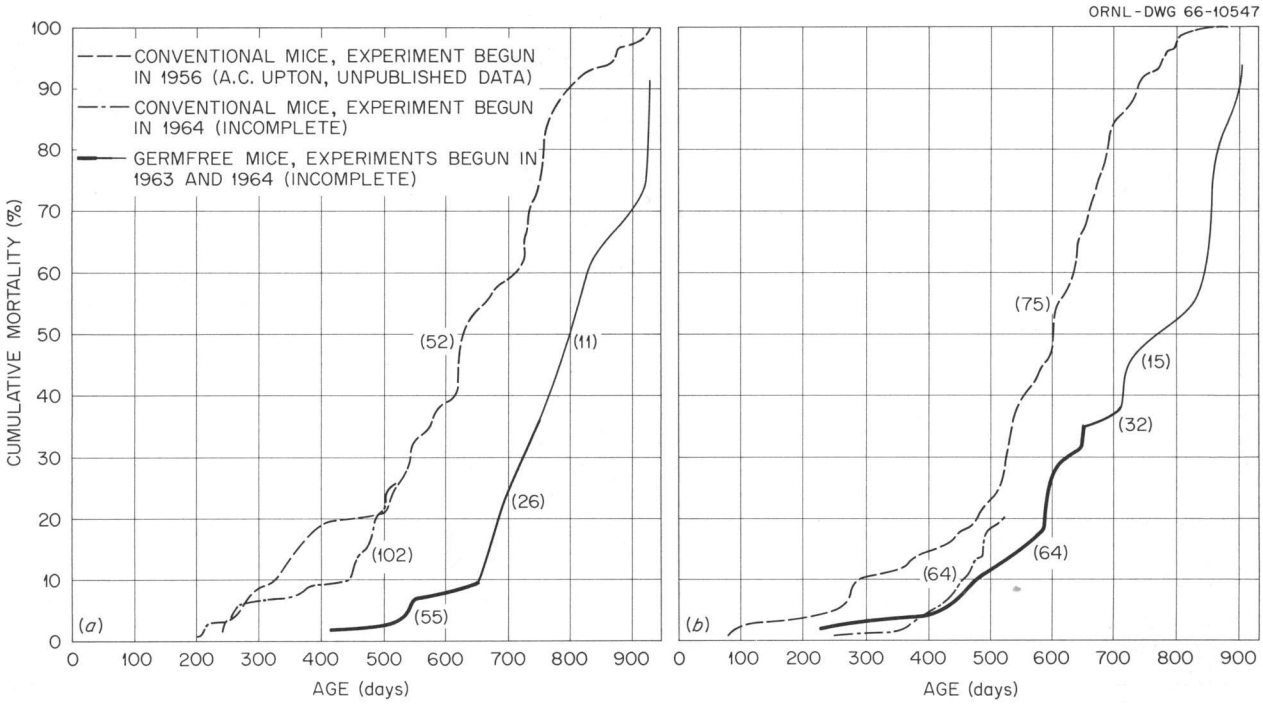

Fig. 11.7.1. Mortality of Untreated RFM Male (*a*) and Female (*b*) Mice as a Function of Age. Number of mice entering experiment in parentheses.

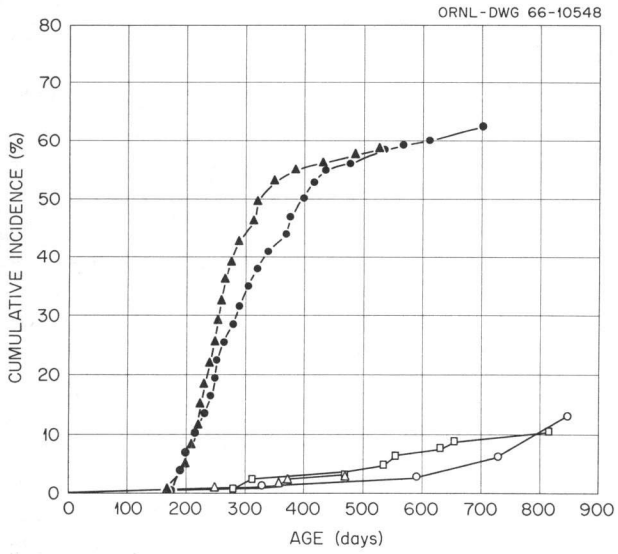

Fig. 11.7.2. Incidence of Thymic and Nonthymic Lymphatic Leukemia in Irradiated and Unirradiated RFM Female Mice as a Function of Age.

▲ Conventional mice receiving 300 r x radiation at five to six weeks of age, experiment begun in 1964 (incomplete).

● Germfree mice receiving 300 r x radiation at five to six weeks of age, experiment begun in 1963 and 1964 (incomplete).

△ Conventional mice, no irradiation, experiment begun in 1964 (incomplete).

□ Conventional mice, no irradiation, experiment begun in 1956 (A. C. Upton, unpublished data).

○ Germfree mice, no irradiation, experiments begun in 1963 and 1964 (incomplete).

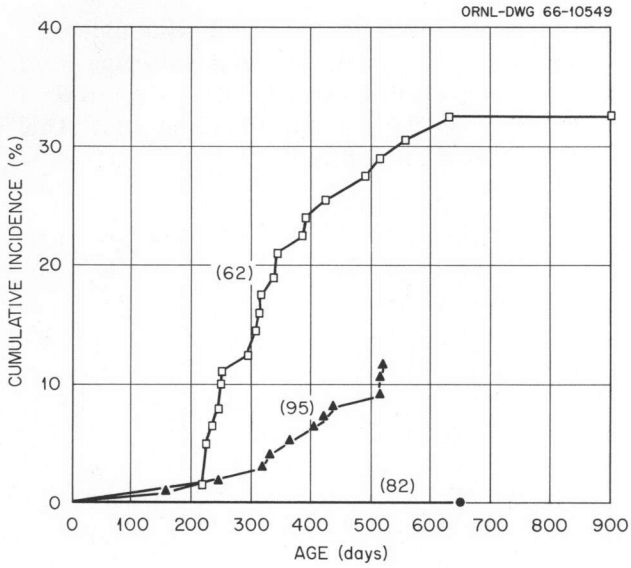

Fig. 11.7.3. Incidence of Myeloid Leukemia in Male RFM Mice Receiving 300 r X Radiation at Five to Six Weeks of Age. Number of mice irradiated in parentheses. □, conventional mice, experiment begun in 1956 (A. C. Upton, unpublished data); ▲, conventional mice, experiment begun in 1964 (incomplete); ●, germfree mice, experiments begun in 1963 and 1964 (incomplete).

As in the previous experiments[1] degenerative diseases were observed in both germfree and conventional mice, that is, glomerulosclerosis, arteriosclerosis, and polyarteritis, but not amyloidosis. Although RFM germfree mice have the enlarged cecum associated with a germfree environment, few deaths due to cecal volvulus, a disease frequently noted in germfree ICR mice, were observed. Auricular thrombosis, which is observed in conventional mice and barrier mice but not in germfree ICR mice, has been observed in aging irradiated and unirradiated germfree RFM mice. The general similarity of widely diverse pathological lesions between germfree and conventional RFM mice suggests that the absence of a detectable microbial flora and fauna alters aging processes and late somatic effects of radiation relatively little, a notable exception being the absence of myeloid leukemia in germfree mice. This exception warrants further study.

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11.8 INHIBITION OF SOLID TUMOR FORMATION BY PRIOR IMMUNIZATION WITH FORMALIZED LEUKEMIC SPLEEN EXTRACTS

R. L. Tyndall
Ernestine Teeter
J. A. Otten
N. D. Bowles

Introduction. – A previous report described cytopathic effects and a transformed growth pattern in an established cell culture (JLS V5) infected with the Rauscher leukemia virus.[1] Subsequent studies have shown that the transformed cells are highly tumorigenic and produce myxofibrosarcomas when injected subcutaneously into newborn BALB/c mice.[2] The present study was undertaken to determine if immunization of pregnant BALB/c with Rauscher virus vaccine could protect the resultant newborn mice against growth of myxofibrosarcomas.

Results and Discussion. – Formalized vaccines were prepared from sodium citrate extracts of leukemic and normal BALB/c spleen cells by the procedure described by Fink and Rauscher.[3] Eight- to ten-week-old female BALB/c mice were injected intraperitoneally with 0.5 ml of a mixture containing equal volumes of Freund's incomplete adjuvant and either Rauscher virus vaccine, control vaccine, or sodium citrate. Five days later the mice were mated. Two weeks after copulation, as judged by the presence of vaginal plugging, the pregnant mice were injected subcutaneously with 0.1 ml of their respective vaccine without adjuvant. One to four days after birth the newborn mice were injected subcutaneously with 1×10^5 cells from either transformed JLS V5 cultures or primary myxofibrosarcomas. In each of two separate experiments, three groups of pregnant mice were immunized as described. One group received the Rauscher virus vaccine plus adjuvant, the second group received normal spleen vaccine plus adjuvant, and the third group received either sodium citrate plus adjuvant or were untreated. The resultant newborn mice in each group were injected subcutaneously with transformed JLS V5 cells in the first experiment and with primary myxofibrosarcoma cells in the second. The combined data from the two experiments show a myxofibrosarcoma incidence of 4/33 (12%) in offspring of mice inoculated with the Rauscher virus vaccine compared with an incidence of 29/42 (70%) in the corresponding control mice (Fig. 11.8.1). These results indicate

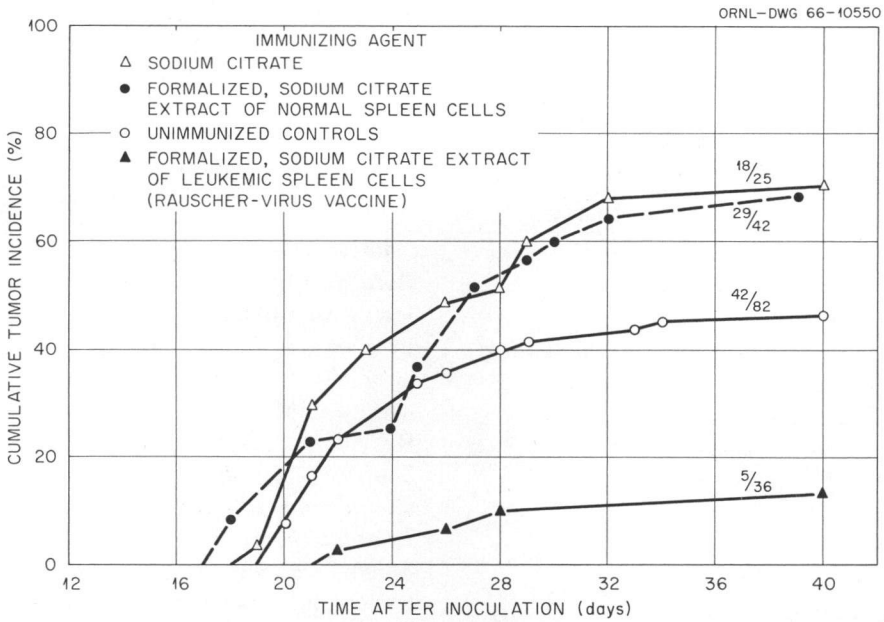

Fig. 11.8.1. Influence of Maternal Immunization on Incidence of Myxofibrosarcomas in Newborn BABL/c Mice.

that immunization of pregnant BALB/c mice with formalized leukemic spleen extract (Rauscher virus vaccine) can subsequently protect the offspring against development of myxofibrosarcomas. Studies are under way to determine if myxofibrosarcoma cells and Rauscher virus possess related antigens and whether other leukemia virus vaccines have a similar effect on the growth of the myxofibrosarcomas.

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11.9 HIGH TITERS OF INFECTIOUS RAUSCHER LEUKEMIA VIRUS PRODUCED IN VITRO

R. L. Tyndall N. D. Bowles
J. A. Otten Ernestine Teeter

Introduction. – Wright and Lasfargues have described an established cell culture (JLS V5) infected with, and continuously propagating, the Rauscher leukemia virus.[1] Originally, the cell-free undiluted tissue culture fluid from such cultures was leukemogenic, but more recently the titer has declined, so that leukemogenic activity can now be obtained only with 100× concentrates of tissue culture fluid.[1] Gross CPE or morphologic transformation in the JLS V5 cultures, grown in either NCTC 109 with human serum or Eagle's basal medium with calf serum, has never been observed by Wright and Lasfargues (personal communication). We have noted, however, that both CPE and morphologic transformation are evident when our infected JLS V5 cultures are grown in Eagle's basal medium with 10% calf serum.[2] We thus initiated this study to determine what factors might be responsible for the differences observed between the two laboratories in regard to the JLS V5 culture.

Results and Discussion. – Since Wright and Lasfargues routinely used trypsin in subculturing the JLS V5 cells, whereas rubber policemen were used in our laboratory, the effects of trypsinization on CPE were tested. Half the cell monolayer from a bottle culture of JLS V5 was detached with a rubber policeman and replanted in NCTC 109, and the remaining cells were detached with the use of 0.25% trypsin solution and replanted in NCTC 109. These two sublines were routinely propagated in both NCTC 109 and BME for six

Table 11.9.1. Titers of Rauscher Leukemia Virus in Culture Fluids from Various Sublines of the JLS V5 Cell Culture

Subline No.	Number of Leukemic Mice[a]/Number of Mice Inoculated[b]				
	Dilution of Culture Fluid				
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
25	5/5	5/9	9/12	ND	ND
68	ND	8/8	10/11	2/4	ND
67	ND	9/10	5/7	8/10	7/12

[a]20/20 mice grossly diagnosed as leukemic were histologically confirmed.

[b]ND = not done.

months using policemen or trypsin, respectively, for each subpassage. During this period CPE and transformation have been evident, both in the culture routinely subsultured with trypsin and in the cells subcultured with policemen.

Since trypsin treatment failed to affect the CPE, virus titrations were carried out to determine if the CPE and transformation could be indicative of increased virus yields. Either NCTC 109 or BME culture fluid from various sublines of JLS V5 cultures was centrifuged to remove cell debris. Tenfold serial dilutions were made of the centrifugates, and 0.1-ml aliquots of each dilution were injected intraperitoneally into newborn BALB/c mice. The mice were observed 90 days for gross signs of leukemia, presumably virus induced. Grossly leukemic mice were autopsied, and the leukemia was histologically confirmed (Table 11.9.1). It is obvious that CPE and transformation are associated with production of high titers of infectious leukemia virus in our cultures, since leukemia can be obtained only by injection of 100-fold concentrates of fluid from JLS V5 cultures devoid of CPE.[1] Studies are under way to determine what factors are responsible for inducing such high yields of infectious virus in our cultures.

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11.10 BACTERIOLOGY OF MICROBIOLOGICALLY DEFINED, AS COMPARED WITH CONVENTIONAL, MICE

R. C. Allen G. W. Fortner
J. H. Chandler, Jr. R. T. Jones
L. J. Serrano

Introduction. – Mice with a defined intestinal flora have been set up for study of the effects of low-level irradiation.[1] These mice are maintained in a barrier facility designed to prevent contamination from the environment. The intestinal flora was established by contamination of germfree RF mice with the following organisms: *Lactobacillus acidophilus, Bacteriodes thetaiatamicron, Clostridium bifermentans, Escherichia coli* 1324, *Proteus mirabilis, Streptococcus faecalis,* and *Staphylococcus albus.* This group of organisms was chosen becuase they represent, in general, the major types of bacteria comprising the intestinal flora of conventional RF mice, that is, mice housed in a facility where the microbial environment is essentially uncontrolled.

To determine the incidence and distribution of bacteria in tissues other than the gastrointestinal tract, barrier and conventional mice have been serially sacrificed. The following tissues were obtained aseptically and cultured for bacteria: lung, heart, liver, spleen, kidney, trachea, uterus or testis, vaginal cervix, and brain. Data were collected on 250 defined mice and 50 conventional mice.

Results and Discussion. – Only rarely were bacteria other than the ones used to establish the intestinal flora in the barrier mice isolated from the tissues of these animals. These were saprophytic forms and are believed to represent contaminants. More than 50% of the tracheal cultures for both barrier and conventional mice were positive. The most prevalent members of the tracheal flora of conventional mice were *Pasteurella pneumotropica* and various species of hemolytic streptococci, neither of which are found in the defined mice. The defined mice have a flora in this region consisting mostly of *Proteus mirabilis* and *Streptococcus faecalis,* which probably represent a replacement phenomenon in the absence of other upper-respiratory-tract inhabitants. A similar difference in predominating types was noted between the two groups of mice with respect to cultures of uterus and vaginal cervix. Lactobacillus species were frequently isolated

from the trachea in both groups. Lung cultures were positive in about 10% of the mice in both groups. The types of bacteria isolated varied, with no predominance of any one type. The positive cultures probably represented contamination from the upper respiratory tract of the animals. No evidence of pneumonia was noted in either group in this particular series. Cultures of heart, liver, spleen, kidney, and brain were negative in more than 90% of barrier mice and 95% of conventional mice. The organism most commonly isolated from these organs was *Lactobacillus*. The significance of the presence of this and other bacteria in these deep tissues remains to be determined.

References

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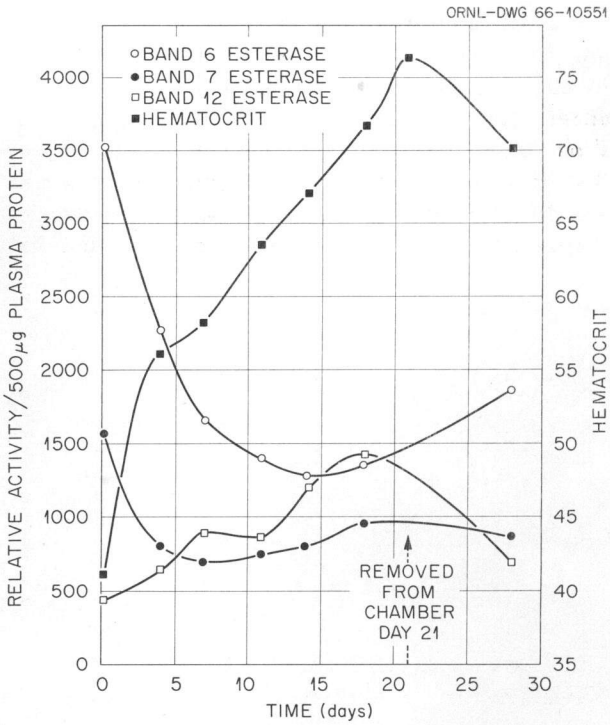

Fig. 11.11.1. Quantitative Plasma Esterase Isozyme Changes in Mice Made Polycythemic by Caging in Hypoxia-Inducing Chambers in Relation to Time After Introduction of the Mice into the Chambers.

11.11 QUANTITATIVE INTERRELATIONSHIPS OF MOUSE PLASMA ESTERASES IN ERYTHROPOIETIN PRODUCTION

R. C. Allen Dorothy J. Moore

Introduction. – On electrophoresis in acrylamide gel,[1] most of the erythropoietin activity of mouse plasma has been located in the region of the testosterone-dependent band-6 esterase isozyme. Slight erythropoietin activity was also found to be associated with the band-7 esterase. In order to determine the possible relationship between esterase and erythropoietin, esterase isozyme activity was studied during erythropoietin production in mice made polycythemic by caging in hypoxia-inducing chambers.[2]

Results and Discussion. – With increasing hematocrit levels, which presumably indicate increased erythropoietin production, the specific activity of the band-6 esterase isozyme markedly diminished (Fig. 11.11.1). A return of esterase activity toward normal started to occur three to five days prior to the peak in the hematocrit level and reached about one-half of normal one week after removal of the mice from the chamber. A similar decrease was noted in band 7; however, recovery of the activity of this band was apparent much earlier. Band-12 esterase isozyme activity, on the other hand, was correlated directly with the hematocrit level.

The inverse relationship between the esterase activity in band 6 and the presumed erythropoietin level suggest that band 6 is acting as a carrier protein in which increased amounts of erythropoietin block esterase sites. A similar situation is suggested from the results of the band-7 isozyme; however, the low erythropoietin activity previously found in this band may have been due to cutting errors in excising band 6. Whether or not band 7 also is actually a carrier protein has not been adequately determined. The rise in band-12 isozyme activity, concomitant with increasing erythropoietin synthesis, suggests a possible role of this esterase in erythropoietin production; however, this relationship is not clear from this study.

It is of interest to note that all these esterases are cholinesterases,[3] based on their sensitivity to eserine, and that isozymes 6 and 12 are also under testosterone control, as is erythropoietin. From these preliminary studies it would appear that two, and possibly three, esterase isozymes are directly involved in erythropoiesis, either as carrier proteins or in the synthesis of erythropoietin.

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[3]R. C. Allen and R. L. Hunter, unpublished.

General Physiology

Radiation Biology (12.1)		Molecular Physiology (12.2)	
G. E. Stapleton[a]	Carol M. Newman[b]	W. D. Fisher	Tom Grizzard F. W. Shull, Jr. Rose Mary Dziak[c] Mary D. Murphy[c] A. W. Prestayko[d]

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12.1 ATTEMPTS TO ISOLATE FROM CELL EXTRACTS SUBSTANCES THAT CONTROL CELL DIVISION IN *ESCHERICHIA COLI*

W. D. Fisher — F. W. Shull, Jr.
G. E. Stapleton — Carol M. Newman[1]

We have previously described a system[2–4] for investigating the biological activity of substances derived from cell-free extracts of *Escherichia coli* which control cell division (cross-septation) in certain strains of *E. coli*. The latter strains are induced by both ultraviolet and ionizing radiation to form long filaments that are nonseptate. Inhibition of cross-septation is more radiosensitive than is macromolecular synthesis in these strains and at low doses of radiation accounts quantitatively for lethality, based on colony formation, since filament formation ultimately leads to cell lysis.

Results and Discussion. – The supernatant from high-speed centrifugation of the cell extracts (150,000 × *g* for 300 min) is inactive in promoting division in irradiated cells, but has now been shown to contain a stimulator as well as a potent inhibitor of cell division.

The stimulatory and inhibitory substances have been separated by ethanol precipitation at pH 4.5. The stimulatory substance is soluble under these conditions. Figure 12.1.1 shows the results of an assay for the separated substances. As shown in curve *A*, the inhibitory substance reduces the survival of x-irradiated cells. Curve *B* shows the reduction of survival by increasing amounts of inhibitor in the presence of a constant amount of stimulator. Curve *C* shows the action of added particulates[4] under the same conditions as in curve *B*. From these and other data we have

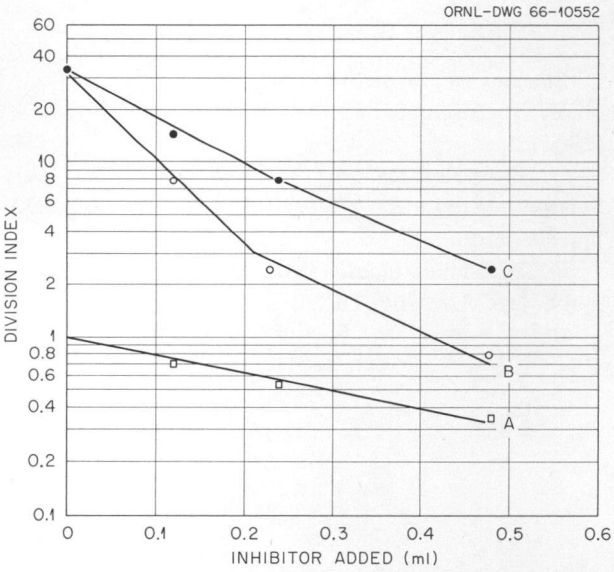

Fig. 12.1.1. Separation of Stimulatory and Inhibitory Substances by Ethanol Precipitation at pH 4.5.

concluded that the high-speed supernatant contains stimulatory and inhibitory material and that the particulate fraction may contain an enzyme which destroys the inhibitor.

It is tempting to postulate that normal cell division in this species may be controlled by the relative abundance of stimulator and inhibitor in the cell during the division cycle. The concentration of the inhibitor would then be enzymatically controlled. Experiments are under way to investigate this possibility utilizing synchronous cultures of *E. coli*.

We have been able to purify the stimulatory substances by gel filtration and ion exchange chromatography. The active material is tenaciously bound by even the weak cation exchangers and requires high salt or pH for elution, as would be expected of a polyanionic substance. Although gel filtration suggested a molecular weight of less than 2000, fictitiously low estimates may be obtained by this type of chromatography for highly charged materials. Paper chromatography of the material stripped from the cation exchanger gave three ninhydrin positive spots, one of which contained all the biologically active material. On this basis we suggest that the stimulatory substance may be a basic polypeptide, and further attempts at isolation will utilize procedures developed for fractionation of peptides derived from hydrolysis of histones by other investigators.[5]

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12.2 ISOLATION AND CHARACTERIZATION OF RIBOSOMES FROM *TETRAHYMENA PYRIFORMIS*

A. W. Prestayko W. D. Fisher

Ribosomes from log-phase cultures of *Tetrahymena pyriformis* have been isolated for the purpose of studying their physical and chemical characteristics. Cells were grown in 1.0% proteose-peptone medium containing 0.1% liver extract. Cells were disrupted in a French pressure cell in 0.02 *M* Tris-HCl buffer pH 7.5, containing 0.0005 *M* $MgCl_2$. Ribosomal components were examined by sucrose gradient centrifugation using a zonal ultracentrifuge.

The ribosomes isolated from the gradient had sedimentation coefficients of 40, 60, 81, and 125*S*. RNA was extracted from each class of ribosomes with buffer-saturated phenol and separated on a 5 to 20% sucrose gradient. These RNA's had sedimentation coefficients of 25, 18, and 13*S*. These RNA molecules were found to differ strikingly in base composition from that typically observed for vertebrate cells. The base composition of *Tetrahymena* ribosomal RNA has a GC content of 42%.

Studies have also been carried out to determine the chemical properties of ribosomal proteins. These proteins were extracted with 8 *M* urea and subjected to acrylamide gel electrophoresis. Differences in protein extracted from 40, 60, and 81*S* ribosomes were observed.

Studies on *Tetrahymena* ribosomes indicate that the dissociation products of 81*S* ribosomes are not identical with the 40 and 60*S* subunits, as has been reported for some mammalian systems.

Cell Physiology

Peter Mazur	Janice J. Schmidt
S. P. Leibo	Patricia F. Davis[b]
J. F. Brandts[a]	W. H. Ramsey[c]

[a]Consultant.
[b]Loanee.
[c]Student trainee.

13.1 FREEZING OF BACTERIOPHAGE T_4B

S. P. Leibo Peter Mazur

We previously suggested[1] that bacteriophage T_4B is inactivated during rapid warming from the frozen state by the osmotic shock caused by the rapid dilution of concentrated solutes that occurs during warming. This suggestion has been tested further by measuring survival of frozen-thawed phage T_4B suspended in various salt or glycerol solutions. Some recent results support this proposed explanation. Others, however, raise serious doubts as to its validity.

The results, in Table 13.1.1, that support the proposed explanation are as follows:

1. The survival of frozen-thawed T_4B suspended in H_2O was higher when dilutions for assay purposes were carried out with broth solutions. This is consistent with the finding that osmotically shocked T_4B phages undergo an additional inactivation resulting from exposure to very dilute salt solutions.[2]

2. The survival of T_4B suspended in 0.003 *M* KNO_3 was higher the lower the minimum temperature of exposure. This could result from the fact that the cooling rates were higher with lower minimum temperatures. Therefore, the phages were exposed to a concentrated salt solution for shorter times during cooling, and survival was higher after warming. This argument also applies to the results observed for T_4B suspended in 0.003 *m* NaCl and 0.006 and 0.572 *M* glycerol.

3. The survival of T_4B in 5.2 *m* NaCl (the eutectic concentration) was about 100% after cooling and warming from −20 and −30°C. This result is predicted from the proposed explanation, since such a treatment would eliminate any solute dilution during warming and, thus, any osmotic shock.

4. The survival of T_4B suspended in glycerol rose markedly when the phages were frozen to a temperature below −60°C, the temperature at which a glycerol-H_2O solution forms a glass. This result is also consistent with the proposed explanation since the solution cooled to −80°C becomes a solid at −60°C, thus preventing any additional penetration of solute into the phage head. Survival under these conditions was, therefore, higher than that found for phage cooled to temperatures above −60°C, at which additional solute penetration could occur, resulting in a more extensive osmotic shock.

On the other hand, data in Table 13.1.1 that are inconsistent with the proposal that osmotic shock is primarily responsible for inactivation of T_4B that results from freezing and thawing are as follows:

5. The percent survival of T_4B frozen in eutectic concentrations of KNO_3, $MgSO_4$, NH_4Cl, and NaCl (except for the results discussed in item 3) was considerably less than 100%, even though osmotic

shock should have been prevented by the use of eutectic solutions.

6. The survival of T_4B suspended in $MgSO_4$ was similar to that of T_4B in NaCl, despite the fact that T_4B in $MgSO_4$ is resistant to osmotic shock, whereas T_4B in NaCl is sensitive.[2]

Therefore, we conclude that although osmotic shock may be a factor, it cannot be the only agent responsible for inactivation of phage T_4B during rapid warming from the frozen state. The full explanation must await further studies.

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Table 13.1.1. Survival of Phage T_4B Suspended in Various Solutions and Rapidly Cooled and Rapidly Warmed from Subzero Temperatures

Solute	Suspending Concentration (*M*)	Eutectic Point (°C)	Minimum Exposure Temperature (°C)	Percent Survival	
				Broth[a]	H_2O[a]
H_2O	0	0	−20	12.2	13.2
			−50	62.2	47.5
			−80	65.9	56.8
KNO_3	0.003	−2.8	−20		11.0
			−30		26.3
			−50		52.1
			−80		57.8
	1.15	−2.8	−20	2.7	
			−30	4.9	
$MgSO_4$	0.003	−3.9	−20	28.0	23.6
			−30	54.7	38.5
			−50	53.6	49.6
			−80	71.1	48.2
	1.4	−3.9	−80	51.5	
NH_4Cl	4.6	−15.	−80	1.0	
NaCl	0.003	−21.1	−20		2.5
			−30		7.7
			−50		24.8
			−80		44.0
	5.2	−21.1	−20	95.4	
			−30	100.0	
			−80	9.7	
Glycerol	0.006	−60[b]	−20	9.2	
			−50	16.9	
			−80	52.9	
	0.572	−60	−20	23.3	
			−30	28.0	
			−50	32.7	
			−80	69.4	

[a]Medium used for dilutions in assay procedure.

[b]Temperature at which the solution becomes a glass.

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

Peter Mazur Janice J. Schmidt

When yeasts are frozen extremely rapidly by being sprayed as droplets into liquid nitrogen,[1] survivals are higher (10 to 30%) than when they are cooled and warmed at more moderate rates of ~500 to 1500°C/min (0.01% survival).[1,2] Our belief has been that the higher survival in the former case results from the fact that the very high cooling velocities produce intracellular ice crystals sufficiently small to be innocuous and that rapid warming merely serves to prevent the growth of the crystals to a damaging size during warming. More recent experiments, however, indicate that the warming rate is even more critical than the cooling rate.

Since no simple way has been devised to measure the cooling and warming velocities obtained with the spray technique, the experiments were performed with 0.01-ml samples of yeast in 0.6-mm-ID glass capillaries. Cooling and warming velocities were measured by means of a 40-gage thermocouple inserted into the capillaries and an oscillographic potentiometric recorder.

The results are shown in Fig. 13.2.1. It will be noted, first of all, that when cells are warmed slowly (closed circles) or rapidly (open circles), survival drops abruptly as the cooling velocity is increased from 10 to 200°C/min. This drop is due to intracellular ice formation. One of the lines of evidence supporting this conclusion is that it is in this range of 10 to 200°C/min that the cells are calculated to become capable of supercooling (dotted curve), and the greater the extent of supercooling the greater the likelihood of intracellular freezing. The new observation is that when cells are warmed ultrarapidly at 40,000°C/min, the abrupt drop in survival is eliminated, and some 10% of the cells survive regardless of the cooling velocity.

One must conclude from this finding that at least 10% of the cells are capable of surviving intracellular freezing if they are warmed sufficiently rapidly. Since increasing the time for warming from −196 to 0°C from 0.3 sec (warming velocity 40,000°C/min) to 9 sec (warming velocity 1400°C/min) caused survival to drop at least 100-fold, we feel that, if changes in the size or form of the internal ice crystals are in fact responsible for injury, the cells must be susceptible to very minute changes.

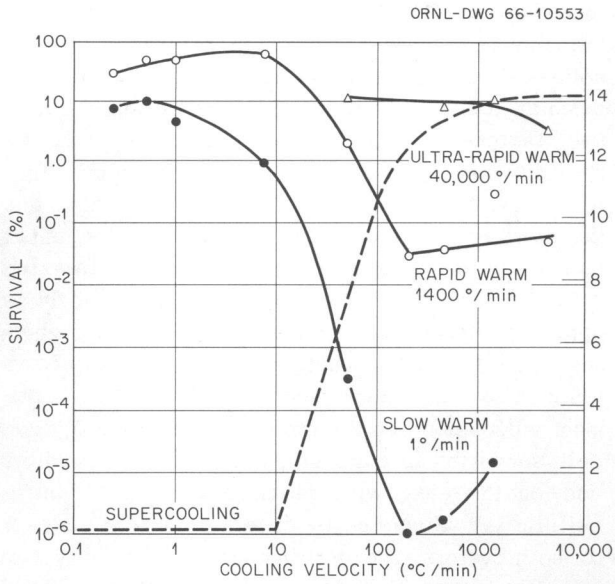

Fig. 13.2.1. Survival and Supercooling of *Saccharomyces cerevisiae* as a Function of Cooling Velocity. Cells were suspended in triple-distilled water, cooled at various rates to −70°C, transferred to liquid nitrogen, and warmed at the rates indicated. The supercooling curve is taken from ref. 3.

There is good evidence that ice crystals can grow and change form during the warming of frozen pure water, solutions, and cells. The suggestion that these changes are responsible for injury is attractive, but the evidence to support it remains circumstantial.

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13.3 EFFECT OF FREEZING ON THE SURVIVAL OF *ESCHERICHIA COLI* B/r

Peter Mazur Janice J. Schmidt
Patricia F. Davis

Introduction. – It has been suggested that most freezing injury can be accounted for in terms of

two factors: (1) the deleterious consequences of the long exposure to the high concentrations of solutes produced during freezing and (2) intracellular ice formation. The contributions of these two factors to injury are affected oppositely by cooling velocity. More rapid cooling lessens injury from solute concentration by shortening the time the cells remain exposed to liquid solutions, but it increases the probability of intracellular freezing with its lethal consequences. As a result of these opposing effects, there should be an optimum cooling velocity. The numerical value of the optimum velocity and the magnitude of survival at the optimum will depend on the rapidity with which a given cell succumbs to exposure to concentrated solutes and on the ease with which it can freeze intracellularly. Since cells differ greatly in respect to both factors, the optimum cooling velocity can and does vary widely from perhaps 0.05 to 3000°C/min.[1,2] The values for the calculated and observed optimum cooling velocity for yeast are given in Sect. 13.2. Both occur at about 10°C/min.

One of the important factors determining the ease of intracellular freezing is the permeability of a cell to water. The more easily it can lose water, the faster it has to be cooled in order to freeze.[3] Since cells of *Escherichia coli* have a much higher surface-to-volume ratio than yeast, they should freeze internally only at higher cooling velocities. Experiments have been initiated to test this hypothesis. In addition, accurate information on the effects of low-temperature variables on the survival of *E. coli* is necessary for evaluating another study on the effects of uv irradiation on frozen cells (see Sect. 13.4).

Methods. – Stationary phase cells of *E. coli* B/r were suspended in 0.005 *M* phosphate buffer, and 0.1-ml samples were frozen to −196°C at rates varying from 10 to 1000°C/min and warmed at rates ranging from 1 to 1400°C/min. Survival was assayed by a standard dilution plate technique.

Results and Discussions. – The first preliminary results are as follows: Survival was affected by both cooling and warming velocity. When the cells were warmed rapidly at 1400°C/min, about 60% survived with cooling velocities ranging from 10 to 500°C/min. But when the cells were warmed slowly at 1°C/min, survivals dropped with increasing cooling velocity; cooling velocities of 10, 40, 500, and 1000°C/min yielded survivals of 73, 54, 20, and 11%. Survival was also affected by the concentration of cells. When cells were cooled at 500°C/min and warmed at 1°C/min, the percentages of survival were 25, 28, 30, 55, and 61% for cell concentrations of 2×10^6, 2×10^7, 2×10^8, 2×10^9, and 2×10^{10}/ml respectively.

These preliminary results indicate that the results with *E. coli* will be analogous to those obtained with yeast, except that the former appear to be less sensitive to cooling velocity.

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13.4 SENSITIVITY OF FROZEN CELLS OF *ESCHERICHIA COLI* B/r TO ULTRAVIOLET IRRADIATION

Peter Mazur W. H. Ramsey

Ashwood-Smith[1] has reported that *Escherichia coli* B/r is as much as seven times more sensitive to ultraviolet irradiation when irradiated while frozen as when irradiated in the liquid state. Sensitivity to x irradiation in contrast is decreased in frozen cells. The greater sensitivity must be due to one of three factors: (1) the organisms receive a higher incident intensity of irradiation, (2) their absorption cross section is higher in the frozen state, or (3) the quantum yield for killing is higher in the frozen state. The first could conceivably occur if multiple scattering by the ice increased the probability of the target being hit. The other two possibilities require that freezing alters the properties of the cells in some way.

Experiments have been initiated using the apparatus described by Füchtbauer and Mazur.[2] Stationary phase cells are suspended in dilute (0.005 *M*) phosphate buffer, frozen to −20 or −70°C at measured rates, and irradiated with a germicidal lamp for various times. Controls consist of frozen unirradiated cells, unfrozen and unirradiated cells,

and unfrozen irradiated cells. Very preliminary results to date indicate that there is little or no increase in sensitivity at −20°C, but a decided increase in sensitivity at −70°C. Thus the existence of an effect appears confirmed, but its explanation must await further experimentation.

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Nucleic Acid Chemistry

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14.1 NUCLEOTIDE SEQUENCES IN TRANSFER RNA

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Identification of Rare Nucleotides. – To establish the chromatographic positions and spectral properties of minor nucleotides that will be met in the sequence work on transfer RNA's, a large-scale (1 g) separation of the nucleotides from mixed *E. coli* transfer RNA's was undertaken. Among the several previously identified minor nucleotides, we found the 2′ and 3′ phosphate isomers of α-cytidine, an anomer of the major component β-cytidine, and also a substance that corresponds to the nucleotide of the alkaline decomposition product of 7-methylguanosine (the presence of thiouridine in *E. coli* phenylalanine transfer RNA we noted previously).[1] The former was first noted in crude yeast sRNA by Gassen and Witzel;[2] the latter, commonly found in chemically methylated nucleic acids, has recently been detected in normal yeast, plant, and mammalian RNA.[3]

Inasmuch as purines methylated in the 7 position are unstable in the dilute alkali commonly used to hydrolyze RNA to nucleotides, 7-methylguanylate will, if present, be encountered as the phosphate esters of 2-amino-4-oxy-5-*N*-methylformamido-6(*N*-β-ribosylamino)-pyrimidine[4] in such hydrolysates (and 7-methylinosinate as the corresponding 2,4-hydroxy analog). We have therefore devoted some time to establish the kinetics of the conversions and the stabilities of the end products under the conditions used in analytical procedures, as well as the positions of all the substances in our nucleoside analytical scheme.[5]

Sequence of tRNA^{Phe} (*E. coli*). – The characterization of tRNA^{Phe} is continuing. There appear to be three internucleotide bonds sensitive to takadiastase RNase T_1 at 37° that are not hydrolyzed at 23° under otherwise identical conditions. This insensitivity to hydrolysis probably arises from regional stabilization of the secondary structure as three of the five guanylate residues in this region are released as free mononucleotides during the 37° hydrolysis. In addition, two peaks of about 10 and 15 residues from the anion exchange chromatography of the 23° hydrolysate are not present in the chromatogram of the 37° hydrolysate.

Analysis of the oligonucleotides produced at 23° shows at least 11 minor nucleotides to be present in tRNA^{Phe}. The composition of tRNA^{Phe} reported earlier[1] is now modified to include these data. There are four residues so far uncharacterized plus 1 Ψ, 1 S, 1 T, 4 5Mec(tentative), 7 (±2) U, 28 G, 13 A, and 15 C. The G and C contents have been confirmed independently by anion exchange analysis. The previous C value[1] was the sum of C and 5Mec. The previous U value is increased because of higher recovery from the oligonucleotide analyses. Phosphate analysis of the oligonucleotides (87 ± 5) confirms our earlier measurement[1] on total tRNA^{Phe}.

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Nucleic Acid Enzymology

Elliot Volkin

Microbial Systems (15.1–15.7)		Developmental Biochemistry (15.8–15.11)	
Elliot Volkin	M. Helen Jones	F. J. Finamore	Rose P. Feldman
W. E. Barnett	Dawn R. Zimmerman	J. S. Clegg[a]	
R. K. Fujimura	Diane M. Dreher[b]	Dorothy S. Cook[c]	
J. X. Khym	Susan M. Elsevier[b]	Sumiko Iyeiri[d]	
C. G. Mead			
J. L. Epler			
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15.1 IMMUNOLOGIC PROPERTIES OF T_2-SPECIFIC PROTEINS

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Julia W. Deitchman[1]

An immunologic approach to the problem of synthesis of new proteins (particularly enzymes) after T_2 infection is being attempted. For this purpose we prepared rabbit antisera to extracts of uninfected and T_2-infected *E. coli* B. The extracts used as antigen represent the total material remaining in the supernatant fraction after centrifugation at 12 Kg for 40 min.

The antisera were first tested against an extract of T_2-infected cells where either leucine-^{14}C or histidine-^{3}H was added to the culture 1 to 2 min after viral infection. About 18% of the labeled protein precipitated with the anti-*coli* B serum, an additional 55 to 60% precipitated with antisera from T_2-infected bacteria, while about 20 to 25% of the labeled protein remained nonprecipitable. Since most of the T_2 virus structural protein had been previously removed from these extracts these data indicate that about half of the labeled protein remaining was "soluble" protein specific to the T_2-infected cell.

The antisera were tested for their ability to inactivate aminoacyl-tRNA synthetases in an attempt to ascertain whether any of these enzymes had (1) been altered as a result of infection or (2) been formed de novo after infection. Excess antiserum was added to partially purified synthetase fractions prepared from uninfected *coli* B and T_2-infected *coli* B. After incubation overnight at 4°C the antigen-antibody precipitates were removed and the supernatants tested for synthetase activity. Appropriate controls using synthetase preparations incubated with control rabbit serum and buffer were included. Eight aminoacyl synthetases (from both uninfected *coli* and T_2-infected *coli*) were not inactivated by either antiserum. Six synthetases (from both sources) were inactivated somewhat more strongly by the uninfected *coli* antiserum than the T_2-infected antiserum. Of the remaining three types of synthetases, the leucyl synthetases were inhibited strongly by very small amounts of both antisera; the lysyl synthetase from uninfected bacteria was not inactivated by either antiserum, but the lysyl synthetase from infected cells was partially inactivated by antiserum from infected

cells; both methionyl synthetases were strongly inactivated by antiserum from infected cells, but very little inactivated by the antiserum from uninfected *coli*.

These results demonstrate that (1) the immunogenic properties of the various synthetases vary considerably, (2) the methionyl synthetase after T_2 infection is strongly antigenic whereas that found in uninfected cells is not. It should be noted, however, that the antibody from infected extracts against the methionyl synthetase is completely cross-reactive with the enzyme of uninfected bacteria. These latter results have been reproduced with another set of rabbit antisera.

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15.2 ON THE REPAIR MECHANISM OF T5 BACTERIOPHAGE DNA MOLECULES

R. K. Fujimura Elliot Volkin

According to a current view the recombination of DNA molecules can occur without DNA synthesis, and rejoining of the strands of DNA molecules occurs by synthesis of a short stretch of polynucleotide between the recombinant. In the presence of sufficient amount of fluoro-deoxyuridine, DNA synthesis is markedly inhibited; thus there is a possibility that most of phosphate incorporation into DNA is for repair of DNA. However, the results showed that most of incorporation is still into newly synthesized DNA. Thus currently, a temperature sensitive polymerase mutant is used at high temperature to inhibit DNA synthesis. There are still several technical difficulties, but the data indicate that repair involving phosphate incorporation occurs in the absence of polymerase.

15.3 ON THE MECHANISM OF INHIBITION OF NUCLEIC ACID SYNTHESES BY COLICINE E2

R. K. Fujimura

According to the present hypothesis, the adsorption of colicine on its specific receptor on the bacterial surface causes some change which somehow inhibits those macromolecular syntheses closely associated with the bacterial membrane. In the case of colicine E2 the primary action is on DNA and the processes associated with DNA. All the other processes seem to be intact. But as reported previously,[1] the viral RNA synthesis of an RNA containing bacterial phage R17, which does not depend on DNA, is also inhibited. Thus, it appears that the viral RNA synthesis is also closely associated with the bacterial membrane. However, before investigating the effect of the change in bacterial membrane on nucleic acid syntheses, I have decided to investigate all the other obvious possible causes.

Viral RNA synthesis is dependent on template RNA, nucleoside triphosphates, and polymerizing enzyme(s). Thus the inhibition could be due to the damage on the template, to inhibition of nucleoside triphosphate synthesis, to inactivation of the enzyme, or to inhibition of the enzyme synthesis. The pulse labeled viral RNA does get somewhat degraded after treatment with colicine, but a considerable amount remains intact. Also, at the late stage of infection when the infected bacteria are treated with colicine and chloramphenicol, there is inhibition of RNA synthesis but no detectable amount of degradation. Thus it is unlikely that the degradation of viral RNA is the cause of inhibition. There is some decrease in synthesis of nucleoside triphosphates; thus the synthesis of one or two of the nucleoside triphosphates may be inhibited. This is still under investigation (in collaboration with D. Dreher, a summer trainee). The inhibition of the synthesis of polymerizing enzyme could not be the cause, since the inhibition of viral RNA synthesis still occurs under the conditions where there is sufficient amount of the enzymes present without additional synthesis of the enzyme. However, inactivation of the preexisting enzyme is still a possibility.

One of the controversial points among the investigators in the field of RNA bacteriophages is the mechanism of viral RNA replication. According to one view, the replication is autocatalytic; that is, newly synthesized RNA serves as a template just as efficiently as the original template. According to another view, the first RNA synthesized is the complementary strand and the template is the double stranded RNA–enzyme complex. The synthesis of double stranded RNA occurs only at an early stage of infection. Thus the RNA

synthesized at a later stage of infection will not serve as the template. If the latter view is correct, there is another possibility that the cause of inhibition by colicine E2 is due to inactivation of the RNA-enzyme complex. Thus it is possible that this study will contribute to settlement of a current controversy on viral RNA replication.

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15.4 PARTITION OF TRANSFER RIBONUCLEIC ACID BETWEEN AQUEOUS SALT SOLUTIONS AND TRICHLOROTRIFLUOROETHANE CONTAINING A QUATERNARY AMINE

J. X. Khym

Introduction. – The partition of transfer ribonucleic acids (tRNA's) between aqueous salt solutions and trichlorotrifluoroethane (Freon) containing a quaternary amine has been shown to be sensitive to slight changes in the concentration of salt in the aqueous phase and also to changes of pH in this phase.[1,2] The effect of the concentration of quaternary amine in the organic phase upon the partition of tRNA's is described here.

Experimental. – The source of tRNA and the extraction procedure have been previously described.[1] Solutions of ten different concentrations (0.01 to 0.5 *M*) of amine in Freon were prepared. Into each of these organic solutions, tRNA was extracted from 0.2 *M* NaCl solutions containing tRNA of concentrations far below the level that saturates the organic phase. Aliquots of each organic phase were mixed with an equal volume of aqueous NaCl solution ranging in concentration from 0.30 to 0.4 *M*. After phase separation, the optical density at 260 mμ of each aqueous phase was compared to controls (0.5 *M* NaCl solutions, which extract essentially all the tRNA from the organic phase). The percentage of tRNA appearing in each of these aqueous phases is given in Fig. 15.4.1. Extraction coefficients for six individual acceptor RNA's were determined after tRNA, initially present in 0.3 *M* amine-Freon, was extracted with 0.32 *M* NaCl; these extraction coefficients are shown in Table 15.4.1.

Results and Discussion. – As shown in Fig. 15.4.1, the percentage of total tRNA found in an aqueous phase at a given concentration of NaCl varies as the concentration of amine in the organic layer is changed. The shapes of the curves in Fig. 15.4.1 suggest that, at a given concentration of NaCl in the aqueous layer, some tRNA's are preferentially extracted at dilute concentrations of amine and more completely extracted by the more concentrated solutions of amine. It appears that other tRNA's are never completely extracted, even at low concentrations of NaCl and concentrated amine solutions. This is substantiated by the single extraction described in the experimental section in which the concentration of amine in the organic phase was 0.3 *M* and the concentration of NaCl in the aqueous phase was 0.32 *M*. For this extraction the E_a^o values given for the six tRNA's in Table 15.4.1 show that phenylalanine and tyrosine tRNA's are essentially completely extracted, while about 90% of the lysine tRNA is found in the aqueous phase. The other tRNA's of Table 15.4.1 are intermediate between these values. By the techniques described here it is possible to separate a given pair of tRNA's by single batch extractions. The separation factor

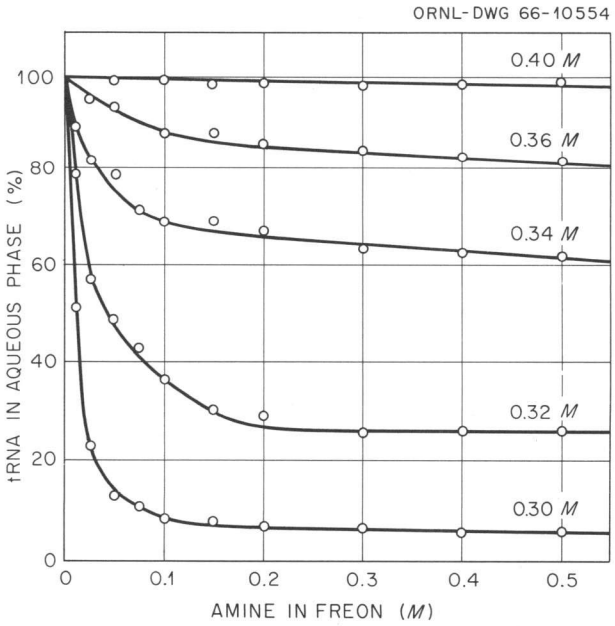

Fig. 15.4.1. Distribution of tRNA Between Different Concentrations of Amine in Freon and Aqueous NaCl Solutions. Percentage was determined by absorbance measurements at 260 mμ.

Table 15.4.1. Extraction Coefficients[a] for Transfer Ribonucleic Acids Determined with 0.3 *M* Amine-Freon Solutions

Organic phase: 10 ml of 0.3 *M* quaternary amine in Freon containing *E. coli* tRNA at 3 mg/ml.

Aqueous phase: 10 ml of 0.32 *M* NaCl. Following mixing and phase separation, tRNA was removed from the organic phase by shaking 8 ml of that phase with 8 ml of 0.5 *M* NaCl.

tRNA Assayed[b]	E_a^o [c]
Lysine	0.1
Valine	0.7
Histidine	4
Serine	22
Tyrosine	60
Phenylalanine	1000

[a]Extraction coefficient (E_a^o) =

$$\frac{\text{Concentration of acyl-tRNA in organic phase}}{\text{Concentration of acyl-tRNA in aqueous phase}}.$$

[b]tRNA in each phase was assayed by measuring ^{14}C-aminoacyl-RNA formation using a given amino acid as previously described.[1]

[c]Percent extracted into organic phase $= \frac{E_a^o}{E_a^o + 1} \times 100$.

(i.e., the ratio of extraction coefficients) for lysine and phenylalanine tRNA's is as high as 10,000 (see Table 15.4.1).

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15.5 FURTHER PURIFICATION OF THE OLIGODEOXYRIBONUCLEOTIDE TRANSFERASE ENZYMES

C. G. Mead

The purification procedure presently used in the preparation of the oligodeoxyribonucleotide transferase system involves three main steps: (1) removal of nucleic acids by passing an extract of T2 infected *E. coli* cells through a DEAE-cellulose column, (2) ammonium sulfate fractionation by back extraction, and (3) passing this fraction through a phosphocellulose column. The final preparation has been contaminated to a variable degree with DNA-exonuclease activities. An additional step has been added that results in a preparation free of DNA-exonuclease activity. This is accomplished by chromatography of the preparation after step 3 on a hydroxylapatite column. The oligodeoxyribonucleotide transferase activity is eluted with a 70 to 90% recovery and a threefold increase in specific activity.

15.6 sRNA'S AND PROTEIN SYNTHESIS

J. L. Epler W. E. Barnett

Initiation. – Recent studies suggest formylmethionyl-sRNA as an initiator of protein synthesis in *E. coli*. Our current work with the fungus *Neurospora crassa* has resolved two methionine-tRNA's and indicated the possibility of a shift in their RNA code words when compared with *E. coli*.

The observations have prompted a study of N-terminal amino acids and formylated derivatives in *Neurospora* crude and purified extracts.

Termination. – The code word UAG has been suggested as a polypeptide chain termination signal. We have attempted to isolate a tRNA that binds to this triplet by preparing and fractionating ^{32}P sRNA. Thus far, the approach has been impractical because of the high background of nonspecific binding encountered.

RNA Code Words. – We have previously reported multiple synthetase, multiple-RNA systems in *Neurospora*. Anticipating a role of these systems in regulatory mechanisms of protein synthesis, we have investigated the coding characteristics of two *Neurospora* phenylalanine-tRNA's. When acylated individually with the appropriate phenylalanine synthetase, the two tRNA's show similar polymer responses in a polynucleotide-dependent ribosomal binding assay.

Large scale separation of *Neurospora* tRNA's is now in progress.

15.7 MULTIPLE AMINOACYL-RNA SYNTHETASE SYSTEMS

W. E. Barnett Susan M. Elsevier
Dawn R. Zimmerman J. L. Epler

Fractionation of cell-free extracts of *Neurospora* and also yeast has revealed the presence of two chromatographically separable aminoacyl-RNA synthetases for L-phenylalanine. In both organisms the multiple synthetases appear to acylate *E. coli* tRNA's in addition to the normal phenylalanine-tRNA. In the *Neurospora* system, each phenylalanyl-RNA synthetase shows a specificity in acylation for one of the two *Neurospora* phenylalanine-tRNA's resolved by countercurrent distribution techniques. No yeast RNA fractionation has been carried out.

Presently, we are involved in isolating and purifying these enzymes, primarily by DEAE-cellulose and hydroxylapatite column chromatography. Numerous properties of the purified enzymes will then be examined.

15.8 P^1, P^4 DIGUANOSINE 5′-TETRAPHOSPHATE AND BRINE SHRIMP EMBRYOGENESIS

F. J. Finamore J. S. Clegg
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The unusual nucleotides P^1, P^4 diguanosine 5′-tetraphosphate and P^1, P^3 diguanosine 5′-triphosphate compose approximately 5% of the dry weight of *Artemia salina* cysts and thus represent a large source of potential energy for embryonic development. However, these pyrophosphate anhydrides undergo neither turnover nor changes in concentration between gastrulation and hatching of the nauplii. After hatching the actively swimming nauplii utilize P^1, P^4 diguanosine 5′-tetraphosphate at a remarkable rate. Indeed our experiments indicate that as P^1, P^4 diguanosine 5′-tetraphosphate is utilized there is a concomitant increase in adenosine 5′-triphosphate in the nauplii.

During early embryogenesis, $NaH^{14}CO_3$ is incorporated equally into 28*S*, 18*S*, and 4*S* RNA components, whereas there is no incorporation of the isotope into DNA until the emergence stage. Exposure of the dry cysts to 50,000 r of x irradiation causes little, if any, alteration in labeling pattern of the DNA and RNA during subsequent development.

15.9 URACIL-CONTAINING COMPOUNDS IN AMPHIBIAN EGGS

Egidio Puccia[1] F. J. Finamore

Introduction. – Previously it was reported that an unusual uracil-containing compound in the acid-soluble fraction is present in amphibian eggs and embryos.[2] Most recently, we have undertaken a comparison of the acid-soluble and oligonucleotide fractions of ovarian eggs to determine the similarities or differences in distributions of the uracil-containing compounds in these preparations.

Materials and Methods. – For three consecutive days, each of 12 females of *R. pipiens* received an intraperitoneal injection of 250 μc of uridine-^{3}H. On the fourth day, the animals were killed and ovarian eggs were taken. The acid-soluble fraction was prepared and subjected to DEAE-cellulose chromatography. The uracil-containing compounds were rechromatographed and analyzed for radioactivity. The nucleic acids were extracted using a hot NaCl procedure, and the oligonucleotides were separated from polynucleotides by acid precipitation. The oligonucleotide fraction was then subjected to DEAE-cellulose chromatography and analyzed for radioactivity.

Results and Discussion. – The specific activities of the uracil-containing compounds in the acid-soluble fraction and the oligonucleotide fractions are shown in Table 15.9.1. The oligonucleotide fraction contains a compound similar to uridine 5-monophosphate, as judged by phosphorus analysis, paper chromatography, and anion exchange chromatography. However, the specific radioactivity of this UMP is considerably lower than

Table 15.9.1. Specific Activities of Uracil-Containing Compounds in Amphibian Eggs

Compound	Specific Activity (^{3}H counts min^{-1} micromole^{-1})	
	Acid-Soluble Fraction	Oligonucleotide Fraction
UMP	882,000	134,500
UDP	977,000	
UTP	1,029,000	

that found in the acid-soluble fraction. Whether the UMP present in the oligonucleotide fraction is due to degradation of RNA or is the result of destruction of a labile compound during extraction of the RNA is currently under investigation.

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15.10 LIFETIME OF RIBOSOMAL RNA IN A NONGROWING TISSUE

Dorothy S. Cook

Turnover of 28*S* and 18*S* ribosomal RNA (rRNA) has been investigated in the integumentary tissue of intermolt (nongrowing) specimens of the land crab, *Gecarcinus lateralis*. During the intermolt period (10 to 12 months) of the life cycle, the number and size of cells remain constant; mitoses and sloughing off of old cells are not observed. Total RNA remains constant. The rate of incorporation of ^{14}C-leucine into proteins of this tissue also remains constant at less than one-sixth the rate observed in the premolt period when the epithelium is actively synthesizing the epi- and exocuticles.[1,2] After a single injection of ^{3}H-uridine, rRNA is rapidly labeled. The specific activity (S.A.) of the 28*S* peak reaches a maximum value in approximately 30 hr. The S.A. remains at the maximum level for more than three weeks, after which time it falls rapidly to approximately one-quarter of the maximum. The rate of decline is greatest at 28 days. 18*S* rRNA labels more slowly but once labeled follows the same time course of breakdown.

Additional observations are: (1) Within 12 hr after injection, counts disappear from the hemolymph; therefore the constant S.A. of rRNA is not being maintained by a continual influx of ^{3}H-uridine from the hemolymph. (2) Injections of large amounts of unlabeled uridine do not significantly change the S.A. (3) Preferential reutilization of ^{3}H-uridine cannot account for the results. It is concluded therefore that rRNA in this tissue is not randomly destroyed but has a well-defined lifetime of 28 days.

The S.A. of integumentary tissue rRNA has not returned to background levels even 80 days after injection of label. Radioautographs indicate that the ^{3}H-uridine is incorporated into both epidermal and blood cells of the integumentary tissue. Experiments are in progress to determine which type of cell contains the stable rRNA.

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15.11 BREAKDOWN AND RE-FORMATION OF SOMATIC MUSCLE DURING THE MOLT CYCLE OF THE LAND CRAB, *GECARCINUS LATERALIS*

Dorothy S. Cook

Growth in Crustacea occurs only in the molting period. In the land crab, *Gecarcinus lateralis*, the principal increase in weight of the whole animal occurs before ecdysis, although the increase in linear dimensions does not occur until after ecdysis.[1] This study was undertaken to determine when the actual growth of muscle occurs, growth being defined as a net increase in muscle protein.

For intermolt (nongrowing) animals the amount of recoverable muscle per claw equals (0.095 ± 0.013) $(\text{propus length} \times \text{propus width})^{3/2}$. During the premolt period (approximately 50 days in large specimens of this animal) the quantity of muscle per chela decreases to approximately half the amount found in intermolt animals; during this time the fraction of protein per unit of wet weight of muscle remains constant. It appears that whole fibers are being cytolized and that the remaining fibers are normal.

Immediately after ecdysis water moves into the tissue. Approximately two weeks later, muscle proteins are rapidly synthesized, and the animal enters a new intermolt period with both the amount of muscle per chela and the dimensions of the chela greater than in the previous intermolt period. The ribosomal RNA used in the postmolt synthesis of muscle protein is formed in the premolt period and retained for more than two months.

References

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Chemical Protection and Enzyme Catalysis

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Jesse James[b] W. A. White[a]

[a]Dual assignments.
[b]Consultant.

16.1 CHEMICAL PROTECTION

D. G. Doherty Margaret A. Turner

The sequential screening procedure we developed for examining compounds for protective activity is useful for selecting those compounds of potential interest. However, in order to make valid activity-structure comparisons, a more quantitative measure of biological response such as the radiation LD 50/30 days is essential. This procedure has been used to evaluate a number of compounds that at a single, lethal 900-r dose gave complete survival and to establish reference values for standard compounds. A computer program has been developed which will accept the raw mouse data and calculate the LD-50 values. All our data have been entered in this program, and the values reported for AET and MEA were obtained over several years of testing in two mouse strains, $1C3F_1$ and $BC3F_1$. All values for other compounds are the results of at least two male and two female LD-50 determinations. Compound doses were the maximum that could be given with no deaths due to chemical toxicity. No significant difference in response was noted for male and female mice.

Both MEA and AET given ip yielded typical dose response curves (Fig. 16.1.1) in that the dose reduction obtained is proportional to the log of the dose of compound administered. An extrapolation of these curves indicates that if the toxicity could be cut in half and twice as much compound given, the increment in LD 50 would be about 400 r. The results obtained with mixtures of glutathione and AET (LD 50, 1700 r) are approximately 100 r better than could be expected from the increased dose of AET, indicating that glutathione also plays a role in protection obtained beyond that of reducing the toxicity of AET. With regard to the disulfides, several reports in the literature indicate that they give the same maximum dose reduction factors as the corresponding thiols. This is clearly not the case

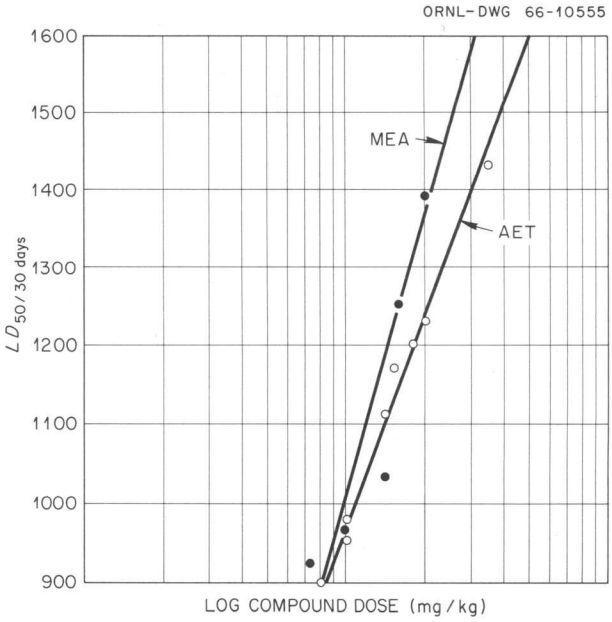

Fig. 16.1.1. Compound Radiation Dose Response of MEA and AET.

Table 16.1.1. LD 50/30 Days of Amino and Guanido Thiols and Disulfides[a]

Compound	Mouse Strain	Intraperitoneal Route			Oral Route	
		Dose (mg/kg)	Dose (m*M*/kg)	LD 50/30 (r)	Dose (mg/kg)	LD 50/30 (r)
Mercaptoethylamine (MEA)	1C3	200	1.76	1390 ± 35	400	988 ± 52
2-Aminoethyldisulfide (AED)	1C3	240	1.06	1220 ± 20	480	1032 ± 19
	BC3	240		1200 ± 20		
Aminopropyldisulfide (APD)	1C3	100	0.40	800 ± 15		
	BC3	100		810 ± 25		
2-Aminoethylisothiourea (AET)	1C3	360	1.28	1390 ± 22	640	1346 ± 10
(2-mercaptoethylguanidine)					800	1342 ± 31
	BC3	360		1427 ± 19	640	1293 ± 18
Guanidoethyldisulfide (GED)	1C3	120	0.30	998 ± 15	240	934 ± 20
	BC3	120		1123 ± 20	240	1073 ± 18
3-Aminopropylisothiourea (APT)	1C3	240	0.82	1290 ± 16	480	1355 ± 41
(3-mercaptopropylguanidine)					640	1355 ± 26
	BC3	240		1268 ± 39		
3-Guanidopropyldisulfide (GPD)	1C3	120	0.28	868 ± 61	240	828 ± 38
	BC3	120		1000 ± 25	240	975 ± 32
None	1C3			689 ± 8		
	BC3			753 ± 6		

[a]MEA as the HC1; AED and APD as the diHC1; AET, APT, GED, and GPD as the diHBr.

Table 16.1.2. LD 50/30 Days of Aminoalkylphosphorothioates[a]

Compound	Dose (mg/kg)	Dose (m*M*/kg)	LD 50/30 Days (r)
2-Aminoethyl-	480	2.68	1500 ± 30
Methylaminoethyl-	600	3.1	1338 ± 18
	300	1.55	991 ± 23
DL-2-Aminopropyl-	800	4.15	1437 ± 15
D-2-Aminobutyl-	480	2.3	1303 ± 22
3-Aminopropyl-	1000	5.2	1450 ± 26

[a]As the monosodium salts.

(Table 16.1.1) in the two strains of mice we have used whether the route was ip or oral; 2-aminoethyldisulfide (crystamine) (AED) was the only one that could be given in equivalent (as-SH) molar doses to the thiol, the others being two to three times more toxic. However, on a *molar basis*, using the values in Table 16.1.1, AED is less effective than MEA, and GED more effective than AET. The addition of glutathione to GED allows the GED dose to be doubled, but the LD 50 is only increased from 1123 r to 1330 r. Thus detoxifying disulfides do not seem to be a reasonable route toward greatly increased protective activity.

As another approach toward compounds of reduced toxicity, a series of phosphate esters of the aminothiols were examined for protective activity, and the results obtained are given in Table 16.1.2. The toxicity of the compounds was the reverse of that of the thiols in that it was less with increasing carbon chain length. Although the LD 50 values are quite respectable, the protective activity on a molar basis is considerably reduced. The preliminary compound dose response for the methylamine ethyl phosphorothioate indicates that it is similar in slope to that of MEA.

16.2 PEPTIDE AND PROTEIN MODIFICATION

D. G. Doherty W. A. White

The x-ray crystallographic method is a powerful technique for the determination of complex structures. Its effective use in large molecules of biological interest depends to a large extent on the ability to incorporate in the molecular structure atoms of high atomic number in relation to the usual C, H, N, O as bench marks for the interpretation of the x-ray diffraction data. Atoms from iodine (molecular weight 126.9) to uranium (molecular weight 238.03) may be used for this purpose and can be introduced by either covalent linkage, salt linkage, or diffusion into the crystal structure. We propose to explore these various routes in an effort to produce crystalline derivatives with heavy atoms in specific places for x-ray diffraction analysis by R. Einstein, of the Molecular Biophysics Group.

Gramicidin A, a linear *N*-formyl pentadecapeptide-ethanolamide, with only one reactive site – the terminal hydroxyl group – was chosen as a model compound for modification and diffraction studies. Although various derivatives of gramicidin have been reported, none have been crystallized or contain a heavy atom such as iodine. Using a commercial preparation of gramicidin (85% A plus B, C, and D as minor components), we have explored the acylation of the hydroxyl group by *o*-, *m*-, and *p*-iodobenzoyl chlorides and *p*-iodophenylsulfonyl chloride. Strong conditions were necessary to produce reasonable yields of an acylated product consisting of a mixture of mono- and diacyl derivatives. These were separated on a silica gel column, and pure derivatives were isolated. The *m*- and *p*-iodobenzoyl gramicidins have been obtained in a crystalline form, and their structures have been substantiated by data obtained from ir, nmr, and ord measurements. A further series of derivatives containing heavy metals are being planned, and initial experiments are in progress.

16.3 THE CATALYTIC PROPERTIES OF PEPSIN

D. G. Doherty Jesse James

Although pepsin was one of the first enzymes crystallized, the exact specificity of this enzyme has never been extensively examined, and good kinetic data exist for only a few substrates of rather restricted structures. Information of this kind is essential for gaining some insight into the nature of the active site of the enzyme, both as to catalytic groups and specificity groups. In addition, it seemed feasible to improve the assay techniques to permit more precise measurements at very dilute substrate concentrations. With such a technique it would be possible to explore the susceptibility of a wide variety of model substrates to pepsin action and obtain reliable kinetic values for those that are hydrolyzed.

As a means of obtaining more soluble peptides at acid pH, a series of peptides have been synthesized with a free basic group on the N terminal acyl group. The method of Harrington has been modified to detect the release of as little as 0.04 micromole of an amino acid, checked with the standard substrate acetyl-L-phenylalanyl-L-phenylalanine, and kinetic constants in agreement with the literature obtained. Two new substrates, nicotinoyl-L-phenylalanyl-L-phenylalanine and *p*-aminobenzoyl-L-tyrosyl-L-tyrosine, are readily hydrolyzed, and kinetic studies are in progress.

Enzymology

G. D. Novelli[a]

Protein Synthesis (17.1–17.5)

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Audrey N. Best[a]
A. P. Pfuderer
E. F. Phares
Lawrence Rosen[a]
Audrey S. Niyogi
C. J. Wust
D. H. Brown[b]
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Enzymatic Mechanisms in Protein Synthesis (17.6–17.8)

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R. L. Pearson[g]
K. R. Isham
J. E. Geltosky[d]
Barbara H. Chastain

Mammalian Cell Regulation (17.9–17.12)

K. B. Jacobson
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J. B. Murphy
L. V. Kaufman[f]

Participation in Cooperative Programs:
AEC-NIH Chemical Carcinogenesis (22.2–22.5)

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17.1 ISOLATION OF MITOCHONDRIAL sRNA

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W. E. Barnett[1]

Introduction. – Evidence has recently appeared which indicates that mitochondria have the capacity to synthesize protein independent of nuclear control.[2] Luck[3] has demonstrated that mitochondria replicate by division from preexisting mitochondria and that they contain DNA unique to this organelle.[4] In addition, these workers have presented evidence for the existence of a mitochondrial DNA-dependent RNA polymerase. Most recently, Rabinowitz *et al.*[5] have shown that ribosomes are a part of the mitochondrial matrix. Thus most of the machinery for making proteins from transcription through translation appears to be present in mitochondria as a part of their "normal" body of constituents. We felt, therefore, that it was feasible to undertake the isolation and characterization of sRNA from mitochondria in spite of the fact that it probably exists only in minute amounts.

To date we have developed a procedure for large-scale preparation of intact mitochondria from the fungus *Neurospora crassa*.

Mitochondria are assayed by the method of Williams;[6] the fraction used contains cytochrome a, b, c, and c-1, in constant proportions. Preliminary work indicates that RNA (total) is present in amounts of 10 μg per milligram of mitochondrial protein. This is in close agreement with that found in yeast mitochondria.[7]

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17.2 STUDIES ON THE PURIFICATION AND CHARACTERIZATION OF tRNA PYROPHOSPHORYLASE

Audrey N. Best G. D. Novelli

Introduction. – We have initiated an investigation into the properties of the enzyme(s) that incorporates AMP and CMP from ATP and CTP into the terminal position of tRNA,

$$\text{tRNA} \text{-----} + \text{ATP} + 2\text{CTP} \rightleftharpoons \text{tRNA} \text{---} \text{pCpCpA} + 3\text{PP}.$$

Although purification of this enzyme has been achieved from a variety of sources, such as *Escherichia coli,*[1] rabbit muscle,[2] and rat liver,[3] the enzyme has never been extensively purified, and it is possible that separate enzymes are required for the addition of AMP and CMP. The actual affinity of the tRNA for the enzyme(s) has not been measured exactly. We plan to purify the enzyme(s) extensively using a standardized tRNA as the substrate. The purified enzyme will be characterized. It will also be of use in the adenosine end-group analysis of various kinds of tRNA.

Results and Discussion. – We have found that partially purified tRNA[4] from *E. coli* can be degraded to a suitable substrate for AMP incorporation by alkaline periodate oxidation and subsequent treatment with alkaline phosphatase.[5] An enzyme preparation from *E. coli* that incorporates AMP (from ATP) into the oxidized tRNA has been prepared according to Berg's procedure.[1] Although the investigation is in the preliminary stages, it appears to be promising, and a large-scale purification is planned.

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17.3 CONFIGURATION STUDIES OF *ESCHERICHIA COLI* POLYSOMES

Peter Pfuderer

The addition of small amounts of actidione to a logarithmic-phase culture of *E. coli* K-12 has been found to produce an increase in the polysome content of the bacteria. This technique was used to furnish a supply of *E. coli* polysomes for further studies. The sedimentation coefficients and distribution of the polysomes in this bacterial system do not change significantly after treatment with aldehyde fixatives, as opposed to the rat liver system previously studied, making it valuable for electron-micrograph studies of the final polysomes.

The large, free polysomes produced by this treatment are being characterized in the cell and as isolated polysomes by biochemical and physical methods. Figures 17.3.1*a* and *b* are micrographs of polysomes isolated by this technique. The general appearance of these large particles is that of a lacy network of several connected strands. The fine structure of the individual ribosomes is shown in negative contrast in Fig. 17.3.1c. The appearance of these particles varies somewhat with the staining, shadowing, and drying procedure used, and this is being investigated further.

PHOTO 85105

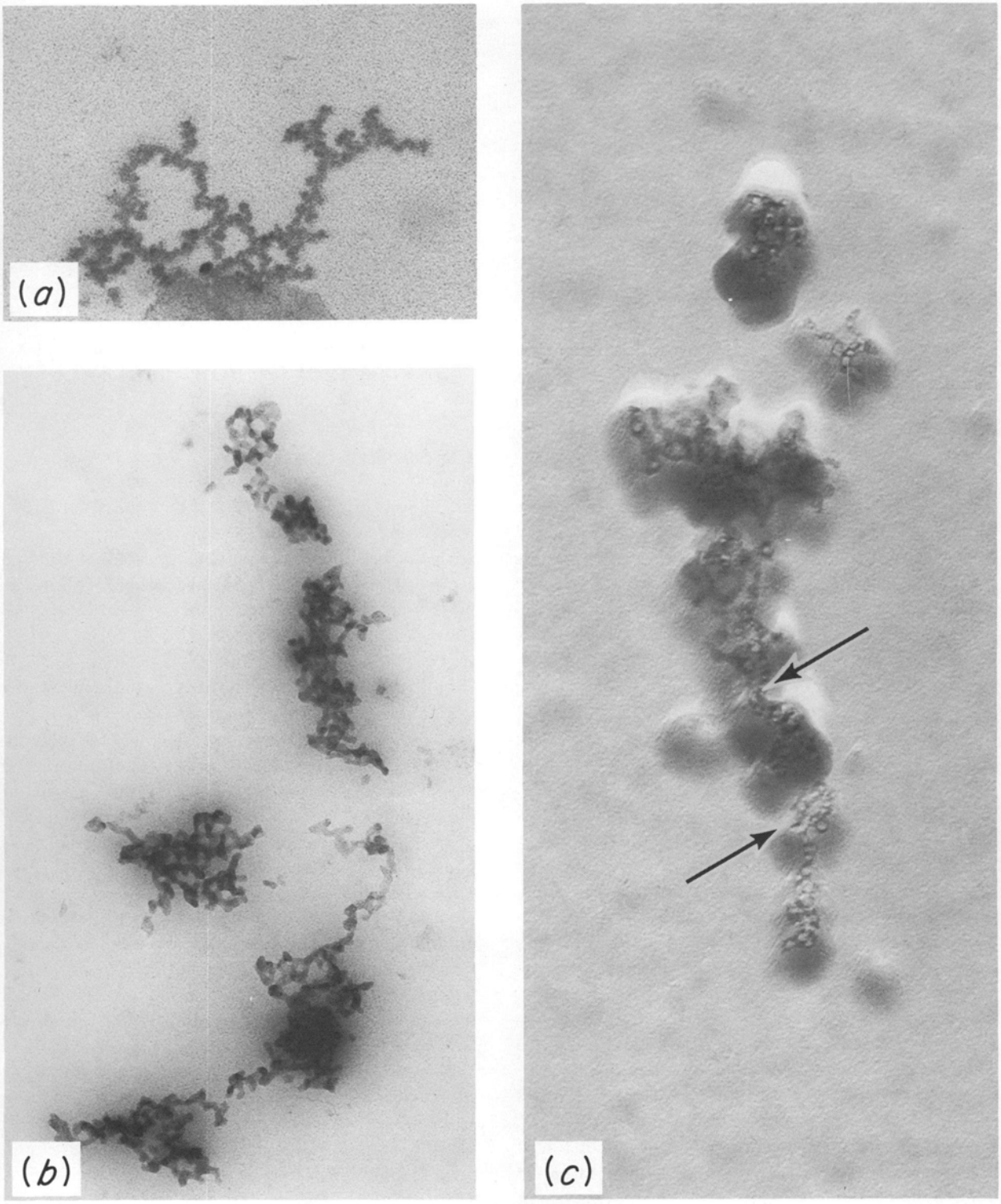

Fig. 17.3.1. Isolated *E. coli* Polysomes Containing Several Strands. *a* and *b*, positive contrast, 73,600×. *c*, negative contrast and shadowed. The arrows show apparent separation into two strands. 132,000×.

17.4 STUDIES ON POLYSOMES IN RAT SPLEEN DURING THE IMMUNE RESPONSE

C. J. Wust D. G. Ridner

Introduction. – In a continuing study to understand the events of the induction phase of antibody biosynthesis on a subcellular level, polysomes of spleens from immunized and nonimmunized rats have been investigated.[1] Certain aspects of this work have been extended and will be reported.

Methods and Results. – The method necessary to prepare polyribosomes from rat spleen homogenates involves $K^+:Mg^{2+}$ molar concentration of 0.1 : 0.01 and the addition of bentonite. It was observed that with one batch of bentonite, 5 mg/ml was optimal to maintain and isolate polysomes. Other preparations of bentonite were made, and a concentration of 5 mg/ml sedimented larger polysomes at 35,000 × *g*. As the concentration of bentonite added to a homogenization mixture was lowered, polysomes were isolated in increasing yields. A concentration of 1.5 mg/ml in the homogenization medium was found to give consistent and reproducible results with several batches of washed bentonite. The finding that amounts of bentonite greater than 2 mg/ml sedimented polysomes is in contrast to the report of Tester and Dure,[2] who studied polysomes from oat seed. They observed that there was selective precipitation of monomeric ribosomes by bentonite.

The method as used allows the release of ribosomes and polyribosomes from endoplasmic reticulum without the aid of detergent. Particles thus obtained, when sedimented through a sucrose gradient by zonal centrifugation, show a ribosomal profile not unlike that found with ribosomes prepared by treatment of microsomes with sodium deoxycholate. Cell-free amino acid incorporation by these preparations is proportional to the relative amount of particles larger than dimers (120*S*). This finding supports our earlier speculation that the monomer (81*S*) from spleen does not incorporate amino acids in a cell-free system.

Degradation of polysomes by ribonuclease, freezing, and thawing or by extended incubation at 0°C (3 hr or longer) results in the conversion of polysomes to 65*S* particles. As this degradation occurs, there is a decrease in the amino-acid-incorporating activity of these preparations. The 65*S* particle is tentatively described as a degradation product or subunit. In vivo incorporation of ^{32}P orthophosphate into the 65*S* particle is not observed unless degradation occurs. Particles smaller than 65*S* have not as yet been found.

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17.5 STUDIES ON POLYSOMES IN SPLEENS OF RATS TREATED WITH ACTINOMYCIN D

C. J. Wust D. G. Ridner

Introduction. – Actinomycin D has been reported to prolong the latent period of the immune response to sheep erythrocytes in both rats and mice.[1] The delay in the immune response was correlated with marked cellular damage, whereas during the recovery period, antibody synthesis, determined by serum titration, was associated with the proliferation of large lymphoid cells, particularly in the germinal centers of the lymphoid follicles in the spleen.[2] The titration of antibody during the production phase revealed an increase of 3 to 4 $\log_2$ units over the response in the untreated immunized animal. It was of interest to study the polysomal population in spleens of animals treated with actinomycin D during periods of cellular damage and during the production phase.

Results and Discussion. – Ribosomal profiles observed after sedimentation through a sucrose gradient by zonal centrifugation may be quantitatively evaluated by planimetry. Preparations from untreated, nonimmunized animals showed the following distribution: 65*S* – 11.5%; 81*S* – 23%; 105*S* – 7%; 120*S* – 38%; 154*S* – 11%; and 175*S* – 9.5%. A similar distribution was found with ribosomes from immunized animals. In spleens of rats three days after treatment with actinomycin, the time of maximal histologic damage, the distribution was as follows: 65*S* – 56%; 81*S* – 26.5%; 105*S* – 7%; 120*S* – 10.5%; 154*S* – 0%; and 175*S* – 0%. It is apparent that polyribosome synthesis is impaired and there is degradation to the 65*S* subunit. Cell-free amino-acid-incorporating ability of these preparations was

proportional to the amount of polysomes found (120*S* or greater). Three days after treatment with actinomycin D, incorporation of labeled amino acid was less than one-third that of untreated animals. The protein-synthesizing capacity was much less in treated, immunized animals than in rats receiving actinomycin only.

During the recovery period from actinomycin treatment, when there was antibody synthesis, the profile was similar to that for animals receiving an immunizing dose only. Incorporation was markedly enhanced, being tenfold more than for untreated, immunized animals. The results agree with the expected changes predicted from histologic and serologic studies. The relatively high incorporating activity of polysomes obtained during the recovery period will be useful in our studies on cell-free antibody biosynthesis.

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17.6 STUDIES ON THE ENZYME RECOGNITION SITE IN PHENYLALANINE tRNA

M. P. Stulberg K. R. Isham

Introduction. – The highly specific transfer of amino acid from enzyme-bound aminoacyl adenylate to tRNA is dependent on a specific recognition site in tRNA for the enzyme. Using highly purified phenylalanyl RNA synthetase[1] and $tRNA^{Phe}$ (ref. 2) from *Escherichia coli*, we are attempting to establish the identity and locus of this recognition site.

Results and Discussion. – Our analysis system consists in testing oligonucleotides for their ability to inhibit the interaction of the synthetase and $tRNA^{Phe}$. If the oligo contains the recognition site sequence, it will demonstrate a competitive inhibition with $tRNA^{Phe}$ for the binding site of the enzyme.

Our procedure for identifying the recognition site consists in testing oligos produced by limited endonuclease hydrolysis of $tRNA^{Phe}$ (ref. 3) for their ability to inhibit the reaction. When a small oligo containing the recognition site has been isolated, its base sequence will be determined to establish its absolute identity. With this procedure we have tested the claim that oligo A is identical to the recognition site in $tRNA^{Phe}$.[4] Our results have been entirely negative; oligo A does not inhibit the interaction of the synthetase and $tRNA^{Phe}$ in our system.

Our procedure for determining the locus of the recognition site in $tRNA^{Phe}$ depends on the limited digestion of $tRNA^{Phe}$ from the 3′-hydroxyl end by highly purified snake venom phosphodiesterase.[5] When the phosphodiesterase has hydrolyzed the $tRNA^{Phe}$ past the recognition site, the remaining residue should no longer be able to inhibit the reaction. By use of phosphodiesterase limit digests produced by incubation with $tRNA^{Phe}$ at different temperatures, $tRNA^{Phe}$ has been hydrolyzed to limit residues of 32 nucleotides. These residues still inhibit the reaction; thus they still contain the recognition site. Digestion down to 15 nucleotides produces residues that no longer inhibit. This suggests that the recognition site lies somewhere between the last 32 and 15 residues in $tRNA^{Phe}$. Presently, we are attempting to pinpoint the locus between these limits. In a related study, we are also attempting to relate the importance of the 3′-hydroxyl adenosine in the binding of $tRNA^{Phe}$ to the enzyme.

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17.7 LARGE-SCALE ISOLATION OF PHENYLALANYL-RNA SYNTHETASE FROM *ESCHERICHIA COLI* B

R. L. Pearson M. P. Stulberg

Introduction. – The collaborative effort between the Biology and Chemical Technology Divisions has proved successful with the isolation of tRNA's.[1] An extension of this effort is now being

directed toward large-scale isolation of specific enzymes. Our first choice for scaleup was phenylalanyl-RNA synthetase. A procedure for the isolation of this enzyme has been reported.[2]

Results and Discussion. – High-speed centrifugation techniques for the removal of ribosomes from bacterial extracts are impractical when dealing with large-scale preparations. We previously investigated the use of porous glass grains for removal of ribosomes by molecular sieving. This method is not feasible at the present time owing to interaction of the soluble proteins with the surface of the glass and to a lack of pore size selection. However, we have solved this problem by adding streptomycin sulfate directly to the supernatants from low-speed centrifugations. This step precipitates the ribosomes and the bulk of the nucleic acids with excellent recoveries of phenylalanyl-RNA synthetase in the soluble phase. Thus we have circumvented a technical problem and in addition have combined two steps into one simple one.

Dialysis in large-scale preparations is another step which is quite impractical. An alternative, the removal of high concentrations of salts by gel filtration through Bio P-10, has proven feasible. High recoveries of the enzyme were achieved.

We have successfully removed precipitates in all steps of the purification with the use of continuous flow through a Sharples centrifuge. The aeration of supernatants by this centrifuge does not denature the enzyme. Thus the time-consuming procedure of batchwise, low-speed centrifugation has been eliminated from large-scale preparation methods.

These modifications of the relatively small-scale procedure developed earlier should allow us to begin production of a large-scale preparation of purified phenylalanyl-RNA synthetase. Concurrently, several other amino-acyl-RNA synthetases will be isolated by further modifications.

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17.8 EFFECT OF METHYLATION ON PHENYLALANINE TRANSFER RNA FROM *ESCHERICHIA COLI*

L. R. Shugart M. P. Stulberg

Two peaks of transfer RNA (tRNA) demonstrating phenylalanine-accepting activity have been isolated by reversed-phase column chromatography from a mixture of crude tRNA obtained after methionine starvation of a "relaxed" mutant of *E. coli*. Initial experiments indicated that both tRNA's differed in their capacities to accept methyl groups and phenylalanine.

Results and Discussion. – Further purification of the two phenylalanine-accepting tRNA's has been accomplished by methylated-albumin column chromatography followed by molecular-sieve column chromatography using P-100 polyacrylamide gel. Experiments[1] utilizing a highly purified phenylalanyl-RNA synthetase indicate that the capacity of the two species to accept phenylalanine is approximately one-half that of a similarly treated "normal" $tRNA^{Phe}$. Preliminary experiments indicate that partial methylation of the undermethylated species increases the capacity to accept phenylalanine. However, since the source of the methylating enzymes is a crude extract of *E. coli*, containing nuclease activity, the isolation and purification of the methylases[2] are being undertaken so that more definitive data may be obtained.

Since differences in activities between normal and undermethylated tRNA's depend on the purity of the two species, we are presently performing more rigorous purification techniques. Hydroxylapatite column chromatography[3] is being employed, since each species has been shown to be contaminated to a lesser, but varying, degree with other tRNA's.

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17.9 ISOZYMES: THEIR REGULATION AND GENETIC CONTROL

K. B. Jacobson E. H. Grell[1]
J. B. Murphy

Introduction. – The existence of multiple forms of an enzyme leads immediately to questions regarding their metabolic significance and the means for the control of the relative amounts of each of these forms. In the case of alcohol dehydrogenase of *Drosophila melanogaster*, the enzyme exists in three electrophoretic forms[2] that are characteristic of the strain of the fly. The genetic control of these multiple forms has been discussed already.[3] Since the genetic analysis suggests that all three forms are controlled at one genetic locus, the purification of the enzymes was undertaken to examine the stability of the individual electrophoretic forms and to test the possibility that the various forms are interconvertible.

Results. – The purification and some of the properties of the alcohol dehydrogenase of Drosophila were discussed previously.[4] Another approach to purification has been taken since the procedure using hydroxylapatite resulted in multiple forms that reequilibrated with one another. This difficulty has been avoided by treating the enzyme with *n*-butanol and adsorbing to DEAE-cellulose. The enzyme, as extracted from the flies, will not adsorb to DEAE-cellulose. Elution of the enzymes yields two main peaks of activity which are separated according to their electrophoretic characteristics.

Discussion. – Further studies on this enzyme will be needed to understand the basis for the multiple forms, but the purification and separation of the various forms, a necessary first step to these studies, has been accomplished.

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17.10 PHYLOGENETIC COMPARISON OF LACTATE DEHYDROGENASE ISOENZYMES IN RODENTS

K. B. Jacobson P. B. Dunaway[1]
J. B. Murphy

Introduction. – The evolution of several protein molecules has been shown by studying the amino acid sequences present in a variety of the vertebrates, for example, hemoglobin, myoglobin, and cytochrome C. The evolutionary relationships of many macromolecules can be detected only in that way, but a preliminary indication of identity or homology can be achieved in the case of lactate dehydrogenase since it exists in 5 to 15 electrophoretically distinguishable forms in vertebrates. Sixteen species of rodents in thirteen genera and three families have been compared in respect to the electrophoretic mobility of the comparable isoenzymes and in respect to the organ-specific patterns (see Table 17.10.1).

Results. – A marked uniformity of the migration rate of LDH-1 exists among the rodents. Of the 16 species, 14 have LDH-1 of identical electrophoretic behavior. The two exceptions are the eastern wood rat, *Neotoma floridana*, mentioned previously,[2] and the golden mouse, *Peromyscus nuttalli*. Four species of *Peromyscus* were examined, and *P. nuttalli* differed from the other three by having a faster LDH-1 and an intermediate type of LDH-5.

The other regular feature of the isoenzymes in the rodents was the regular occurrence of LDH-5 as the only, or predominant, form in erythrocytes. Again this generalization has two exceptions among the 16 species in that LDH-2 and LDH-3 were the main forms present in the chipmunk, *Tamias striatus*, and in the eastern harvest mouse, *Reithrodontemys humulis*.

Discussion. – With regard to the molecular aspects of evolution, the LDH isoenzymes may prove an interesting group of enzymes to examine. Among 16 species of rodents there is a remarkable uniformity of electrophoretic behavior. The exceptional behavior of *P. nuttalli* may indicate that a reexamination is required of the criteria for its original generic assignment. The exceptional erythrocyte LDH types of *R. humulis* and *T. striatus* may provide some insight into an area of isoenzyme control that has remained unexplained, namely the high degree of control exerted by each organ.

Table 17.10.1. Characteristics of Isoenzymes of Lactate Dehydrogenase[a]

Rodent		LDH–1	LDH–5	Erythrocyte	Heart
Scuridae					
Eatamias	Chipmunk	Normal	Mouse		1,2,3(4,5)
Tamias striatus	Eastern chipmunk	Normal	Intermediate	2,3	1,2,3(4,5)
Tamiasciurius hudsonicus	Red squirrel	Normal	Intermediate	5	1,2,3
Cricetidae					
Clethrionomys gapperi	Red back vole	Normal	Intermediate	5	1,2,3(5)
Reithrodontemys humulis	Eastern harvest mouse	Normal	Intermediate	3,2(4,1)	1,2
Sigmodon hispidus	Cotton rat	Normal	Intermediate	5,4	1,2,3
Baiamys taylori	Pigmy mouse	Normal	Intermediate	5,4	1,2
Neotoma floridana	Wood rat	Fast	Intermediate	5,4,3(2)	1,2,3(4)
Peromyscus polionatus	Old field mouse	Normal	Mouse	5	1(2)
Peromyscus leucopus	White-footed mouse	Normal	Mouse	5	1,2(3,4,5)
Peromyscus nuttalli	Golden mouse	Fast	Intermediate	5(4)	1,2,3(4,5)
Peromyscus maniculatus	Deer mouse	Normal	Mouse	5	1,2,5(3,4)
Microtus pinetorum	Pine vole	Normal	Intermediate	5	1,2,3(4,5)
Oryzomys palustris	Rice rat	Normal	Mouse	5	1,2,3(4,5)
Muridae					
Mus musculus	House mouse	Normal	Mouse	5	1,2,3,4(5)
Rattus norvegicus	Norway rat	Normal	Rat	5	1,2,3,4(5)

[a]Intermediate signifies an electrophoretic mobility of LDH–5 faster than the rat and slower than mouse. The numerals refer to the LDH isoenzymes observed.

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17.11 INHIBITION OF VALYL-tRNA SYNTHETASE BY POLYRIBONUCLEOTIDES

Virginia L. Holten K. B. Jacobson

The nature of the interaction between an aminoacyl-tRNA synthetase and the tRNA molecule to which the enzyme transfers the activated amino acid is largely unknown at the present time. In order to account for the high degree of specificity of the reaction for both the amino acid and the tRNA to which the amino acid becomes attached, the theory was proposed that the synthetase possesses a recognition site corresponding to a certain nucleotide sequence in the tRNA. Inhibition of RNA binding to this site could be achieved by tying up this region with a nucleotide sequence similar to that found in the specific tRNA. In the work undertaken this hypothesis was tested by studying the decreased binding of

^{14}C valine to a partially purified tRNA from *Escherichia coli* which was enriched in valine acceptor activity. The enzyme preparation employed in these studies was a partially purified valyl-tRNA synthetase from the same source.[1]

Fifty-three synthetic polyribonucleotides of known content but unknown chain length and sequence were screened for their ability to inhibit the incorporation of labeled amino acid into the tRNA. Preliminary screening experiments indicated that polymers containing various combinations of A, C, and U would inhibit the charging reaction from 10 to 30%. In order to explore this interaction more fully, the polymer poly-ACU (1:1:1) (Miles Laboratory) was selected because it appeared most promising from the standpoint of inhibitory ability and maximum combination of bases.

Although the poly-ACU was not a potent inhibitor of the valyl-tRNA charging reaction (10 to 30%), kinetic analyses consistently indicated competitive inhibition by the polynucleotide with respect to the tRNA. However, when the polynucleotide was exhaustively dialyzed vs sodium chloride (0.5 *M*) or ethylenediaminetetraacetic acid (EDTA) (0.001 *M*), its inhibitory ability was lost, and the optical density decreased by 50%. Gel filtration of poly-ACU resulted in only one ultraviolet-absorbing peak, with recovery of 90% of the absorption and complete retention of the inhibitory ability. These apparently diverging results have not been reconciled at this time.

Recent studies of leucyl[2] and tryptophanyl[3] tRNA's implicate the importance of magnesium in maintaining the amino-acid-accepting ability of tRNA. Thus the valyl-tRNA synthetase reaction was run at limiting and optimal magnesium concentrations, and the inhibitory effectiveness of poly-ACU was assessed under both conditions. Under the former condition (10^{-4} *M* Mg^{2+}, 10^{-4} *M* ATP), poly-ACU inhibited the reaction completely, whereas when the magnesium was optimal (10^{-3} *M* Mg^{2+}, 10^{-4} *M* ATP), inhibition ranged from 10 to 30%. Moreover, the inhibitory ability was retained even after the hydrolysis of the poly-ACU into its component monophosphates. The conclusion then follows that whatever is inhibiting the charging reaction is probably not nucleotide in nature but is a contaminant from either the preparation of the polynucleotide or its extraction and isolation.

Since the inhibition by poly-ACU was more pronounced under conditions of limiting magnesium, the possibility remained that it was attributable to metal removal. Accordingly, the valyl-tRNA synthetase reaction was reexamined in the presence of the metal chelator EDTA. Inspection of the resulting double reciprocal plots showed that EDTA also appeared to be a competitive inhibitor with respect to tRNA.

Thus from these studies a contaminant of the poly-ACU preparation is probably responsible for the competitive inhibition of the valyl-tRNA synthetase reaction. The nature of this inhibitor is not known, but it was observed that EDTA can cause inhibition which is judged competitive by the usual criterion. Thus in the search for an inhibitor which competes with a specific tRNA for the active site of its corresponding synthetase, the criterion that it be of a competitive nature is clearly insufficient.

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17.12 A TEST OF tRNA AS AMINO ACID ADAPTOR IN HEMOGLOBIN SYNTHESIS

K. B. Jacobson

Introduction. – A means of attaching phenylalanine to a tRNA molecule which ordinarily is specific for valine was afforded by the discovery of the specificity of the phenylalanine synthetase of *Neurospora crassa.*[1] Thus the product phe-$tRNA^{val}$ may be used to test the adaptor hypothesis, which states that the positioning of an amino acid in a peptide chain is determined by the structure of the tRNA to which it is attached and is not determined by the structure of the amino acid itself.

Methods. – Ribosomes from rabbit reticulocytes were allowed to synthesize hemoglobin in the presence of ^{14}C-phe-$tRNA^{val}$ and ^{14}C-phe-$tRNA^{ala}$ In a control experiment, ^{14}C-phe-$tRNA^{phe}$ donated

the radioactive amino acid. For reference, ^{3}H-phenylalanine was incorporated into hemoglobin by intact reticulocytes. The ^{14}C and ^{3}H products were mixed, and the globin was hydrolyzed with trypson. The ^{14}C and ^{3}H were determined after chromatographic separation of the resulting peptides.

Results. – In the case of ^{14}C-phe-tRNA^{val,ala} the phenylalanine did not enter the normal positions of the hemoglobin chain. In the control, ^{14}C-phe-tRNA^{phe}, the phenylalanine did enter all but two peptides, but it also appeared in two peptides that are not present in normal hemoglobin. In this later case the two abnormal peptides produced suggest a dual recognition phenomenon, such as may result from the presence of two code-specific types of phenylalanyl-tRNA in *Escherichia coli*. One of these would respond to some other coding sequence.

Conclusion. – These experiments are consistent with the adaptor hypothesis for the function of tRNA in determining the site of an amino acid in a growing peptide chain.

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Biochemical Regulation

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18.1 METABOLIC TURNOVER OF RAT LIVER TYROSINE TRANSAMINASE: EFFECTS OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS

F. T. Kenney Joseph Kendrick
L. E. Roberson

Introduction. – Previous estimates of the half-life of the inducible enzymes of rat liver have been largely made from the rate of loss of enzyme activity in the postinduction phase of hydrocortisone induction. Half-life of tyrosine-α-ketoglutarate transaminase was estimated to be about 2 hr by this technique.[1] Several laboratories, including our own, have observed that inhibitors of RNA and protein synthesis (actinomycin, puromycin, cycloheximide), which effectively block induction of this enzyme, have essentially no effect on the base level of enzyme. Inability of actinomycin to block basal enzyme synthesis has been interpreted[2,3] as indicating template stability, but no explanation is available for the failure of the inhibitors of protein synthesis to cause the expected drop in enzyme level. Since knowledge of enzyme turnover is central to understanding the mechanisms regulating enzyme levels in the mammalian organism, we have undertaken a study of the turnover of tyrosine transaminase and are attempting to resolve the apparent discrepancies in the effects of these inhibitors.

Results and Discussion. – In order to measure half-life in the noninduced, steady-state condition, we have employed "chase" experiments wherein the rate of loss of enzyme radioactivity is measured while labeled enzyme is replaced by "cold" enzyme being synthesized from a pool which is devoid of radioactivity. A single injection of high specific activity ^{14}C-leucine results in labeling of liver proteins for a period of about 1 hr, after which the hepatic leucine pool is no longer labeled. During the period between 1 and 5 hr after isotope administration, tyrosine transaminase radioactivity was determined by immunochemical methods previously described.[3] The points obtained yielded first-order curves in two separate experiments, and the measured half-lives were 1.3 and 1.4 hr respectively. These experiments establish that turnover is rapid in the steady-state condition; indeed, it is even faster than was previously thought.

In experiments in which the rate of transaminase synthesis was measured by "pulse" labeling, we have established that both puromycin and cycloheximide (inhibitors of protein synthesis) block the synthesis of this enzyme almost completely. The enzyme level, however, remained constant or dropped only slightly (*ca.* 25%) in the 4 hr treatment period employed. The known rate of turnover (above) predicts a loss of *ca.* 87% of the enzyme activity in this interval if enzyme synthesis is blocked completely and enzyme degradation pro-

ceeds at the normal rate. These results suggest that degradation as well as synthesis is blocked by inhibitors of protein synthesis; this is now being tested experimentally, as is the effect of actinomycin on enzyme turnover.

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18.2 HORMONAL CONTROL OF ENZYME LEVELS IN THE REUBER HEPATOMA IN VITRO

F. T. Kenney L. E. Roberson
R. L. Tyndall[1] Joseph Kendrick

Introduction. – We have previously described[2] experiments which demonstrate that hormonal induction of enzyme synthesis can be elicited in cell cultures of the minimal deviation Reuber hepatoma. Both hydrocortisone (10^{-6} *M*) and insulin (10 m units/ml) were found to be effective inducers, as in rat liver in vivo. In the present series we have extended these observations to include yet another hormone, glucagon. In addition, we have initiated a study of the effects of these hormones on RNA synthesis in the cultured hepatoma cells.

Results and Discussion. – Glucagon was found to be an effective inducer in adrenalectomized rats (see Sect. 18.7, this report), causing a fivefold elevation of hepatic tyrosine transaminase within 3 hr of hormone treatment. Addition of glucagon (10 μg/ml) to the culture medium elicited an entirely analogous induction of this enzyme in the hepatoma cells. As in the in vivo experiments, when optimal levels of the inducing hormones are employed their effects do not appear to be additive. The culture cells thus provide a system in which these hormonal inductions can be studied independently of other hormones, which to be done in vivo requires that the test animals be hypophysectomized, adrenalectomized, and pancreatectomized. At present these results with cultured cells permit the conclusion that each hormone acts directly on hepatic cells, and not via some other organ or tissue.

Changes in RNA synthesis in the course of induction by hydrocortisone were studied by addition of ^{14}C-orotic acid (5 μc/ml) to cultures with and without hydrocortisone. The steroid effected a 20 to 30% increase in the rate of RNA labeling, in contrast to the 200 to 300% change seen in in vivo.[3] This result raises the possibility that the specific changes in synthesis of RNA associated with enzyme induction may be more easily detected in this system, as they are apparently not rendered undetectable by very large and perhaps unrelated changes in total RNA synthesis. These analyses will be continued, with particular emphasis on the use of ^{32}P as a means of determining the nature of the RNA responding to hydrocortisone.

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18.3 REPRESSION OF ENZYME SYNTHESIS BY GROWTH HORMONE

F. T. Kenney Joseph Kendrick
L. E. Roberson

Introduction. – In previous studies[1,2] it was found that administration of stressing agents to adrenalectomized rats elicited a specific repression of the synthesis of hepatic tyrosine transaminase. These agents were ineffective in hypophysectomized rats, but repression could be elicited in hypophysectomized rats joined parabiotically to adrenalectomized rats given the stressing agent. These indications of a pituitary involvement led to the finding that the hypophyseal growth hormone (somatotropin) could replace stressing agents and was effective in hypophysectomized as well as adrenalectomized animals. However, as was mentioned previously,[2] the repression response has proven increasingly difficult to elicit reproducibly, forcing us to evaluate a number of experimental parameters in attempts to develop conditions permitting further analysis of the repression phenomenon.

Results and Discussion. – Bioassay of growth hormone preparations is tedious and time consuming, so we have employed gel filtration on Sephadex columns to ensure that our preparations have the correct molecular weight. By this technique, we found that several commercial preparations of "somatotropin" (which were inactive in repression studies) contained little or no growth hormone; one such preparation was eluted in the same volume as ATP, which we use as a low-molecular-weight marker. The preparations distributed by the Endocrinology Study Section (NIH) are eluted in single peaks of the correct molecular weight (*ca.* 40,000) and are sometimes, but not always, active in eliciting repression.

Since insulin (an inducer of tyrosine transaminase) was shown to block repression by growth hormone, and is known to be secreted as a result of growth hormone treatment, we used rats made diabetic by alloxan administration as a test system. The test animals must also be adrenalectomized to avoid glucocorticoid induction, which introduces new complications. These animals were found to respond somewhat better than those only adrenalectomized, but were still refractory in several experiments, although they were severely diabetic by blood glucose assay.

Other parameters such as dosage and mode of administration of the hormone, age and nutritional status of test animals, etc., have been investigated, but none have proven to be responsible for the erratic nature of the repression response. We are currently investigating perfusion of isolated livers as a potential solution to this problem.

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18.4 RNA SYNTHESIS IN FETAL RAT LIVER

W. D. Wicks

Introduction. – Preliminary studies on the pattern of RNA synthesis in fetal rat liver have recently been completed. As described in a previous report, four different RNA fractions have been isolated from the livers of 20-day-old fetal rats by the modified thermal phenol-extraction technique. These fractions have been identified by various criteria as ribosomal RNA, transfer RNA, ribosomal precursor RNA, and DNA-like RNA respectively. Among the criteria employed were chemical and isotopic yield (at intervals of 15, 30, 60, and 240 min after the injection of ^{32}P), sedimentation behavior, chemical and isotopic base compositions, kinetics of isotope incorporation, and in the case of transfer RNA (tRNA), the ability to accept amino acids.

Results and Discussion. – The kinetics of labeling of the fetal liver ribosomal RNA were about the same as those observed with adult rat liver ribosomal RNA. The yields of RNA, sedimentation behavior, solubility in 1 *M* NaCl, chromatography on DEAE paper, and base compositions were also analogous to adult ribosomal RNA.

Fetal liver tRNA did not exhibit any turnover of the pCpCpA terminus, in contrast to the adult, where considerable turnover occurs. To test the possibility that the terminal nucleotides of fetal tRNA were undergoing turnover but were removed during the isolation procedure, a sample of adult tRNA from rats which had been exposed to ^{32}P for 60 min was added to a homogenate of liver from fetal rats not given isotope. The combined tRNA's were then isolated by the usual technique, and the ^{32}P remaining in the terminal nucleotides of the mixed tRNA preparation was measured by calculating the percent of ^{32}P in 2′, 3′ CMP after alkaline hydrolysis. There was no difference in the percent of ^{32}P in the CMP of the original adult tRNA and that reisolated with unlabeled fetal liver, showing that the terminal nucleotides of fetal tRNA had not been removed during the isolation procedure. Studies on amino acid acceptor ability showed no difference between adult and fetal tRNA, further verifying the functional integrity of fetal tRNA. All other properties of fetal and adult liver tRNA's were identical.

All the properties of the RNA extracted at 45° with phenol and 0.14 *M* NaCl are consistent with it containing the precursors of ribosomal RNA. It incorporated a great deal of ^{32}Pi during the early labeling intervals, but as the time of exposure to isotope increased, the amount incorporated into this RNA declined relative to the total incorporated into all the RNA fractions. The labeling characteristics of ribosomal RNA were just the reverse, a result consistent with a precursor-product relationship. The early labeled RNA in this fraction

sedimented at 45*S* and 35*S* in glycerol density gradients, but as time passed these gradually diminished while new peaks at 28*S* and 18*S* appeared. The isotopic and chemical base compositions of this RNA and ribosomal RNA were essentially the same, lending further support to the conclusion that this RNA fraction contains the precursors of ribosomal RNA.

The DNA-like RNA (dRNA) was extracted at 80° with detergent added in addition to salt and phenol. Although its chemical and isotopic base compositions were radically different from those of the ribosomal precursor RNA, its kinetics of labeling were similar. This suggests that the newly labeled dRNA is undergoing rapid turnover and being broken down without passing into a stable form such as ribosomal RNA. The labeling kinetics indicate that the rate of breakdown of dRNA is rapid at first but slower later on. If this fraction contains messenger RNA, then it would appear that a spectrum of messenger stabilities exists. At present, studies on the ability of dRNA to direct protein synthesis using a fetal liver ribosome preparation are under way. Preliminary results have shown that the fetal liver ribosome population is largely in the form of polysomes.

Recently a new RNA fraction was obtained from fetal liver when the residue remaining after the usual successive phenol extraction was heated at 100° with 10% NaCl. This RNA was ribosomal-like chemically, but isotopically its base composition was unusual, with a preponderance of ^{32}P in UMP and CMP. It could not be extracted by heating at 100° with 0.14 *M* NaCl, phenol, and detergent and thus must be tightly bound to some cellular component. Although comprising less than 1% of the total RNA, it exhibited the highest specific activity of all the RNA fractions at all intervals (up to 4 hr) after ^{32}Pi was administered. The nature of this RNA is under investigation.

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18.5 INDUCTION OF TYROSINE-α-KETOGLUTARATE TRANSAMINASE IN FETAL RAT LIVER BY HYDROCORTISONE

W. D. Wicks

Introduction. – Previous work has shown that the level of tyrosine transaminase in fetal rat liver is less than 10% of that in the adult at all stages of gestation.[1] Within 12 hr after birth, however, the activity of this enzyme rises to about twice the adult level and gradually declines to the adult level within a day or two. Adrenalectomy at birth inhibits the elevation of transaminase activity but hydrocortisone overcomes this inhibitory effect. Attempts to increase enzyme activity in utero with hydrocortisone or other agents effective in elevating enzyme activity in the adult were uniformly unsuccessful. In view of the recent finding in this laboratory of repression of tyrosine transaminase synthesis and the role of the pituitary in this process,[2] it was decided to reinvestigate the noninducibility of the enzyme in utero.

Results and Discussion. – The first possibility to be investigated was that the fetal pituitary, in some fashion perhaps analogous to the stressed adult pituitary, was secreting some agent(s) which repressed the synthesis of transaminase almost completely. Hypophysectomy might thus be expected to allow enzyme induction by hydrocortisone. The fetal pituitary was removed by decapitation, a technique which has been in use for many years; the fetuses tolerate this procedure quite well, and up to 90% survival has been observed. The administration of hydrocortisone by way of the mother did, in a few instances, lead to dramatic increases in the activity of tyrosine transaminase in the livers of the decapitated fetuses. The nondecapitated littermates generally responded only slightly if at all. In one case, the specific activity of the enzyme went up 20- to 30-fold within 4 hr after hormone was administered. These results show that the normal adult capacity for synthesis of the transaminase exists prior to birth and suggests that some agent(s) from the pituitary normally restricts enzyme synthesis. Hydrocortisone apparently then is able to stimulate enzyme synthesis

in decapitated fetuses in a fashion similar to that in the adult. The frequency of induction in these experiments, however, was quite low and variable in degree. It would seem that some other factor(s) is also involved in the regulation of enzyme activity in the fetus. The most likely source of this factor(s) is the placenta, which is now being investigated.

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18.6 STIMULATION OF TRANSFER RNA SYNTHESIS BY HYDROCORTISONE

W. D. Wicks Terryann Gurney

Introduction. – We have reexamined the selectivity of the approximately twofold increase in transfer RNA (tRNA) synthesis in rat liver which occurs 2 to 3 hr after hydrocortisone is administered.[1]

Results and Discussion. – Adult rats were injected with either 10 mg of hydrocortisone or with saline. The hormone treated rats were all given ^{3}H-orotic acid 60 min later, whereas the control animals received ^{14}C-orotic acid. The rats were killed after an additional 60 min, and the livers from control and treated rats were pooled and frozen in liquid nitrogen. Transfer RNA was isolated by the technique of Brunngraber[2] and then chromatographed on a reverse-phase column[3] at 37°. The eluted fractions were concentrated, and aliquots were taken for counting and for amino acid acceptor analyses. Using six different amino acids, it was found that separation of different tRNA's had taken place and a few multiple peaks were observed (phenylalanine and threonine). The ratio of ^{3}H to ^{14}C in the uncharged RNA column fractions was found to be essentially constant over the whole 200-tube chromatogram. These results indicate little or no selectivity in the stimulation of tRNA synthesis by hydrocortisone. Using the ^{3}H to ^{14}C ratio in UMP isolated from the soluble fraction as a base, a 60 to 80% elevation in tRNA synthesis had taken place 60 min after hydrocortisone was given.

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18.7 STUDIES ON THE MECHANISM OF INSULIN AND GLUCAGON INDUCTION OF TYROSINE-α-KETOGLUTARATE TRANSAMINASE IN RAT LIVER

D. D. Holten F. T. Kenney

Introduction. – Greengard and Gordon[1] have reported a threefold increase in the level of tyrosine-α-ketoglutarate transaminase in the livers of adrenalectomized rats following the injection of pyridoxine. Investigations in this laboratory[2] have shown that the induction by pyridoxine was inhibited by actinomycin D and resulted from an increased rate of synthesis of the enzyme. This suggested that pyridoxine initiates events which increase the rate of transaminase template formation at the transcriptional level. A similar mechanism of transaminase induction has been proposed for hydrocortisone.

The discovery that insulin also induced tyrosine transaminase in rat liver[2] suggested the possibility that pyridoxine induction was secondary to a release of insulin from the pancreas of these animals. The failure of pyridoxine to induce tyrosine transaminase formation in diabetic rats was consistent with this possibility. The fact that the insulin which we had been using contained glucagon caused us to test glucagon as an inducer for tyrosine transaminase. This hormone was also found to increase the transaminase level. This finding necessitated a reinvestigation of the insulin induction of the transaminase using an insulin preparation free of glucagon.

Results and Discussion. – Investigations with insulin free of glucagon have shown that insulin

indeed caused induction of tyrosine transaminase. The induction reached a peak at 3 hr and resulted in an increased rate of incorporation of ^{14}C amino acids into the enzyme. Glucagon at a dose of from 150 to 200 μg per 100 g rat caused a maximal induction of the enzyme. The induction by glucagon also reached a maximum 3 hr after the injection of the hormone. The induction of tyrosine transaminase by glucagon was inhibited by actinomycin D, as was the induction of the enzyme by insulin.[2]

From these results it appears that both insulin and glucagon cause a rapid induction of tyrosine transaminase which requires the synthesis of RNA. The magnitude and duration of the transaminase induction by these two protein hormones were similar but were less than the magnitude and duration of induction caused by hydrocortisone.

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18.8 THE EFFECT OF IONIC STRENGTH UPON THE IN VITRO INACTIVATION OF TYROSINE-α-KETOGLUTARATE TRANSAMINASE FROM RAT LIVER

D. D. Holten F. T. Kenney

Introduction. – We have recently described the instability of apotyrosine transaminase in vitro and its protection by pyridoxal phosphate from inactivation at pH 8.7 and 37°C.[1] It was concluded that the apoenzyme was inherently unstable under these conditions and that pyridoxal phosphate protected the enzyme by combining with the active site on the enzyme. The extent of inactivation of the enzyme in Tris buffer as a function of pH suggested that the ionization of a sulfhydryl group on the enzyme resulted in the instability of the apoenzyme and that pyridoxal phosphate prevented either this ionization or an irreversible inactivation resulting from the ionization. Inorganic phosphate at much higher concentrations also prevented inactivation of the apotransaminase.

Results and Discussion. – An attempt to confirm the results previously reported using a second buffer resulted in the discovery that salt solutions of moderately high ionic strength were able to prevent inactivation of the apotransaminase. Sodium or potassium chloride at a concentration of about 3×10^{-2} *M* afforded half maximal protection of the enzyme from inactivation at 26°C and pH 8.7. Under the same conditions of temperature and pH pyridoxal phosphate afforded half maximal protection at a concentration of 8×10^{-7} *M*, a concentration at which any ionic strength effect would be negligible. The K_m for pyridoxal phosphate in the tyrosine transaminase reaction was found to be 7×10^{-7} *M* in Tris buffer at pH 8.7. The similarity between the concentration of pyridoxal phosphate required for either protection from inactivation or activity in the transaminase reaction confirmed the previous conclusion that pyridoxal phosphate protects by combining with the active site of the enzyme.

At constant ionic strength, increasing the pH from 7 to 9.2 resulted in a general increase in enzyme instability due to increasing hydroxide ion concentration rather than a monoionic titration curve with a p*K* implicating a sulfhydryl group.

Tyrosine transaminase is easily freed of pyridoxal phosphate, and this enzyme undergoes a rapid turnover in vivo (see Sect. 18.1, this report). Alanine-pyruvic transaminase and other transaminases cannot be separated from bound pyridoxal phosphate,[2] and these enzymes undergo a much slower turnover in vivo.[3] The finding by Linderstrom-Lang[4] that denaturation of proteins renders them more sensitive to proteolytic attack suggests that the rates of degradation in vivo, presumably a proteolytic process, may depend in large part upon the conformational stability of a protein. The nature of the binding of pyridoxal phosphate by transaminases may be an important determinant of turnover time in vivo, as it is of stability in vitro.

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Microbiology

Electron Transport Mechanisms in Microorganisms

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19.1 MALIC-LACTIC TRANSHYDROGENASE

M. I. Dolin

The characteristic properties which differentiate malic-lactic transhydrogenase from all previously described pyridine-nucleotide dehydrogenases have been summarized.[1] This investigation is being continued, since the transhydrogenase offers a means for determining the manner in which the chemical environment at specific binding sites can alter the catalytic activity of pyridine nucleotide coenzymes.

Malic-lactic transhydrogenase forms a stable, nonfluorescent complex with its keto acid substrate, pyruvate. Stopped-flow experiments (performed in collaboration with Dr. P. Strittmatter at Washington University School of Medicine) have shown that the complex is kinetically competent since (1) the rate at which the fluorescent enzyme (E·NADH) combines with pyruvate to form the nonfluorescent complex (E·NADH·pyruvate) and (2) the rate of transhydrogenation between E·NADH·pyruvate and malate, to regenerate E·NADH, are rapid enough to be part of the overall catalytic cycle.

To compare these results with those obtained at physiological concentrations of enzyme, a study of the steady-state kinetics of the system was begun. It was necessary first to investigate the kinetics of the spontaneous keto $\rightleftharpoons$ enol isomerization of oxalacetate under a variety of conditions. With this information, and with the aid of differential equations derived by Dr. R. K. Clayton, the conditions necessary for valid rate assays in the direction of oxalacetate formation were determined.

The kinetics of transhydrogenation indicate that malate and pyruvate compete with each other for at least one binding site. An empirical rate equation has been deduced from the experimental data, and it has been shown that rate equations of this general form can be derived for various model two-substrate reactions if the mechanism includes abortive complexes between reduced enzyme and malate and oxidized enzyme and pyruvate. It is of interest that the second-order rate constant for reaction of E·NADH with pyruvate estimated from steady-state kinetics (6.8×10^4 liters M^{-1} sec^{-1}) approaches the value of the constant determined in the stopped-flow studies (8×10^4 liters M^{-1} sec^{-1}).

Further kinetic studies, along with other experimental approaches, will be necessary in order to determine whether the enzyme contains more than one substrate binding site per molecule of bound NADH.

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Plant Physiology and Photosynthesis

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Plant Physiology (20.1–20.5)

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20.1 CHARACTERIZATION AND MODIFICATION OF THE RADIATION-INDUCED, SEED-HYDRATION-RESISTANT ESR SIGNAL IN LETTUCE

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Pat C. Kerr

Introduction. – We showed previously that the ESR signal induced by gamma irradiation of lettuce seeds can be separated into two components: (1) one that can be reversibly eliminated by seed wetting-redrying and reinduced by reirradiation, and (2) one that persists after seed wetting.[2] This report is concerned with further characterization of these signals and also with the possibility of regulation of the radiation-induced, water-resistant signal by nontoxic chemicals that might permit us to see if changes in this signal correspond to any changes in biological responses to radiation.

Results and Discussion. – An experiment was performed to test the possibility that the radiation-induced signal observed after the hydration and redrying might be due to incomplete wetting of the seed. Irradiated seeds and unirradiated controls were shaken in flasks of water for as long as 24 hr. For all shaking times studied we found essentially the same radiation-induced signal after seed wetting and redrying. By contrast, the radiation-induced, water-sensitive signal is completely eliminated within the first hours of wetting. These results do not rule out the possibility that after the seeds have finished their water uptake there may still be certain hydrophobic molecular sites not reached by the water. But the results do show that the radiation-induced signals that seem to persist after wetting the seeds cannot be explained by suboptimal imbibition times.

The dependence of numbers of radiation-induced spins (C_1, spins per unwetted seed, and C_2, spins per wetted-redried seed) on dose (D in megarads) can be empirically described by

$$C_{1,D} = C_{1,\infty}(1 - e^{-kD}) = 7.4 \times 10^{14}(1 - e^{-0.50D})$$

and

$$C_{2,D} = C_{2,\infty}(1 - e^{-\lambda D}) = 5.8 \times 10^{13}(1 - e^{-0.17D}).$$

In these equations $C_{1,\infty}$ and $C_{2,\infty}$ represent the apparent saturation values (in spins/seed) approached at very high doses, and the products ($kC_{1,\infty}$ and $\lambda C_{2,\infty}$) represent the efficiency of spin production (in spins seed^{-1} megarad^{-1}) at low doses, where $C_{1,D}$ and $C_{2,D}$ are proportional to dose. The different values of the constants k and λ indicate physical differences between the radiation-induced unpaired electrons in unwetted vs wetted-redried seeds. Although we cannot state whether this indicates different radical species rather than other differences in the environment of the unpaired electrons, the different kinetics of induction extend our previous report concerning differences in power saturation for the radiation-induced signals in unwetted vs wetted-redried seeds.[2]

Treatment of irradiated seeds with iodide solutions reduced the radiation-induced, water-resistant signal in a manner similar to the previously reported reduction in endogenous signal.[3] These actions of iodide could be replaced by treatment of wetted seeds with I_2 vapor. Thus it might be suggested that perhaps I_2 itself, liberated from iodide solutions, might be the agent effective in reducing the ESR signals. The I_2 treatment, however, tended to produce two undesirable effects: (1) seed toxicity, and (2) appearance of another ESR signal, characterized by a derivative curve peak-to-peak width approximately twice as great as the endogenous and radiation-induced signals we have studied. No such complicating effects were observed with the iodide treatments, except perhaps for osmotic inhibition of germination at high concentrations of iodide.

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20.2 SEPARATE AND OPPOSITE ACTIONS OF GAMMA IRRADIATION ON IN VIVO CHLOROPHYLL DESTRUCTION IN LIGHT AND IN DARKNESS

A. H. Haber Carolyn Scurry
R. Z. Gehring[1]

Introduction. – Irradiation generally accelerates senescence in plants and animals. Such well-known radiation effects as (*a*) decreased and earlier cessation of growth, (*b*) inhibition of cell division, and (*c*) chromosomal imbalance resulting from chromosome breakage in dividing cells should be expected to contribute to the accelerated senescence. This report describes a study of the effects of acute doses of gamma irradiation on senescence of detached apical sections of wheat leaves floated in petri dishes. The developmental pattern of the wheat leaf is such that both cell division and cell growth have ceased in this portion of the leaf well before the plant is one week old. Therefore, the radiation effects (*a, b, c*) mentioned above cannot contribute to any radiation-induced senescence that may occur in this simple system, which is also free of any abscopal effects of radiation. The criterion of senescence used is the deterioration of chloroplasts, whose loss of integrity is indicated by decreasing chlorophyll content despite favorable illumination.[2]

Results and Discussion. – When leaf tips are cut from one-week-old plants, senescence occurs over a period of the next several weeks under 16-hr photoperiods (33,900 ergs cm^{-2} sec^{-1} white light). Although the time course of this senescence can be altered by various chemical treatments,[2] there is no effect resulting from acute radiation doses as high as 100 kilorads, which dose does drastically affect growing wheat plants. Acute exposure to 500 kilorads produces complete chlorophyll destruction within a few days. At an intermediate dose, 250 kilorads, the leaf tips consist of two populations: one which resembles those given 500 kilorads, and one which resembles those given 0 or 100 kilorads except for a slightly faster rate of senescence.

The enhanced destruction of chlorophyll by very high doses was shown to result from a gamma-radiation-induced increase in sensitivity to photodestruction. This conclusion followed from experi-

ments in which some leaf tips were kept in darkness following irradiation. In darkness after gamma irradiation there was a decreased rate of chlorophyll distruction relative to non-gamma-irradiated controls. The gamma-ray doses were the same as those that produced an increased rate of chlorophyll destruction in the light. It remains to be seen whether these actions of gamma irradiation on leaf tips subsequently kept in light or in darkness represent a separation of physiologically significant processes or merely reflect consequences of lethality. These findings do show, however, that massive actue doses as high as 100 kilorads, which have drastic effects on growing wheat plants, are ineffective on this simple system in which senescence can be studied rather directly. These findings suggest that senescence per se is remarkably resistant to radiation when uncomplicated by radiation-induced inhibition and earlier cessation of growth, mitotic inhibition, and genic imbalance resulting from chromosome breakage in dividing cells. A similar conclusion on the ineffectiveness of 100 kilorads on roots, after radiation-induced mitotic inhibition itself prevents chromosome imbalance, is given in the immediately following report (Sect. 20.3).

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20.3 PREVENTION OF RADIATION-INDUCED ROOT MERISTEM COLLAPSE BY RADIATION-INDUCED MITOTIC INHIBITION

D. E. Foard Sarah A. Angel

Introduction. – The absence of cell lethality and histological abnormalities in gamma-plantlets (wheat seedlings grown from heavily irradiated dry grain) has been attributed to the radiation-induced absence of nuclear and cell division during seedling growth.[1] Our findings on gamma-plantlets thus far are consistent with this idea. In order to test the idea further, we have irradiated both growing gamma-plantlets and seedlings growing from unirradiated grain and compared them histologically. We have begun such studies with a histological comparison of lower seminal root apices.

Results and Discussion. – Unirradiated seedlings and gamma-plantlets (800 kilorads) of similar sizes (three and four days old respectively) were chosen for the experiments. At these times the lower seminal roots are histologically immature. A group of these unirradiated seedlings and gamma-plantlets were exposed to 50 or 100 kilorads of gamma rays, an exposure that in previously unirradiated plants induces chromosome breakage detectable in dividing cells. Another group of these unirradiated seedlings and gamma-plantlets received no seedling irradiation and thus served as controls. After root growth had ceased in the irradiated seedlings (8 to 11 days later), the plants were fixed and histological sections prepared. The results are as follows:

1. Dose – 0 kilorads to dry seed, 0 kilorads to growing seedling: Roots appear normal, that is, many mitoses occur in apical region; cells and tissues have typically immature features.

2. Dose – 0 kilorads to dry seed, 50 to 100 kilorads to seedling: Roots are very abnormal. The root tips are necrotic, that is, cells of the root cap and root apex proper appear collapsed and devoid of contents. Maturation of tissues occurs close to the root apex proper.

3. Dose – 800 kilorads to dry seed, 0 kilorads to seedling: No necrosis or collapse at the root apex or elsewhere. Each of the cells and tissues has the appearance of typical, mature cell and tissue types.

4. Dose – 800 kilorads to dry seed, 100 kilorads to seedling: The roots appear indistinguishable in all respects from those described for dose 3.

From the above descriptions it is clear that the only plants having roots with necrotic areas are those which received 0 kilorads to the seed and 50 or 100 kilorads to the seedling. The point of interest is that the damage occurs in the root apex proper, the place of most active mitotic activity. On the other hand, those plants which received 800 kilorads to the dry seed, a dose sufficient to inhibit mitosis, did not have necrotic root tips, even though they received 100 kilorads as seedlings. It should be emphasized that 50 kilorads seedling irradiation produces necrosis in previously unirradiated plants, whereas 100 kilorads seedling

irradiation produces no noticeable effect after 800 kilorads seed irradiation. These observations are consistent with the idea that the abnormalities found in the growing zones of irradiated roots are due to chromosome imbalance resulting from chromosome breakage in dividing cells. Furthermore, the observations are consistent with the idea that the absence of mitoses after irradiation prevents such abnormalities, even though the chromosomes themselves undergo much radiation-induced breakage. A similar conclusion on the ineffectiveness of 100 kilorads on leaf tissue, after naturally occurring mitotic inhibition, is given in the immediately preceding report (Sect. 20.2).

References

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20.4 HISTOLOGIC ASPECTS OF NUCLEAR LABELING OF *VICIA FABA* ROOTS EXPOSED TO ^{3}H-THYMIDINE FOR SHORT TIMES

D. E. Foard John McLeish

Introduction. – Despite the numerous studies of short-time labeling of *Vicia faba* root nuclei with ^{3}H-thymidine, there have been no published histological studies necessary to uncover any heterogeneity of labeling due to different rates of incorporation of ^{3}H-thymidine in different zones of the root. Evans[1] exposed *Vicia faba* primary roots to ^{3}H-thymidine for various intervals and found that in squashes of the apical 2 mm the percentage of interphase nuclei scored as labeled after exposures of 5, 10, and 20 min was similar, 25 ± 5%, whereas after exposures of 30, 40, or 60 min the percentage was 41 ± 6%. The present experiment has been performed to see whether or not these differences could be due to different rates of incorporation of ^{3}H-thymidine in different zones of the root. For this purpose sections instead of squashes have been used, so as to be able to determine where labeled nuclei occur in the root. Evans' methods otherwise have been followed where possible.

Results and Discussion. – The beans were soaked in tap water for 48 hr. Thereafter, seed coats were removed and the seeds planted in vermiculite. The plants were used when the primary roots were about 6 cm long. The primary roots were exposed to an aerated solution of ^{3}H-thymidine (2.0 curies per millimole) at a concentration of 3 μc/ml. The apical centimeter of each of five roots was fixed in freshly prepared CRAF solution after 5 min, the apical centimeter of each of five roots after 20 min, and the apical centimeter of each of five roots after 60 min exposure to ^{3}H-thymidine. The roots were stained with the Feulgen reagent and were prepared for embedding in paraffin. The roots were sectioned, and autoradiographs were prepared with NTB emulsion. The slides were exposed to the emulsion for either three or six weeks at 4°C.

There was no or negligible labeling of nuclei in any region of roots exposed to ^{3}H-thymidine for 5 min, even when the slides were exposed to the emulsion for six weeks. In roots exposed to ^{3}H-thymidine for 20 min, labeled nuclei appeared in the periphery of the apical 2 mm, that is, in the epidermis and in the 2 or 3 outermost layers of the ground meristem. There were no labeled nuclei in the procambial region of any of the roots studied. A preliminary count of nuclei in the apical 2-mm region shows that only 10% of the interphase nuclei are labeled. From 3 to 10 mm back of the tip a larger percentage, about 40%, of interphase nuclei are labeled, including nuclei in procambial cells. In roots exposed to ^{3}H-thymidine for 60 min, labeled nuclei occur in all tissues of the apical 2 mm of the root. A preliminary count of interphase nuclei shows that in the apical 2 mm approximately 50% of the interphase nuclei are labeled. Up to 70% of the interphase nuclei are labeled in the zone 3 to 10 mm back of the tip.

These results differ from Evans' findings in two main respects: (1) We have found no significant labeling in interphase nuclei of roots exposed to ^{3}H-thymidine for 5 min and (2) the percentages of labeled interphase nuclei in roots exposed to ^{3}H-thymidine for 20 and 60 min differ from the percentages in the corresponding roots studied by Evans. The present results suggest that ^{3}H-thymidine is taken up and incorporated first in the region of the root several millimeters back of the apical region and in the peripheral layers of the apical region (apical 2 mm) itself. These results are of interest because they suggest different rates of incorporation of ^{3}H-thymidine in different zones of the root, an important but

hitherto neglected aspect of the interpretation of short-time labeling experiments.

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20.5 NUCLEIC ACID SYNTHESIS IN INTERPHASE NUCLEI OF *VICIA FABA*

John McLeish

Introduction. – Nuclei isolated from root meristems of various higher plant species, including polyploid series, have wide differences in DNA content, but these are not matched by proportional differences in the amounts of nuclear RNA.[1] Within species, on the other hand, parallel changes in the amounts of DNA and nuclear RNA have been found to accompany cellular differentiation in the growing nonmeristematic regions of the root.[1] The possibility that there may be parallel changes in the amounts of DNA and RNA in interphase nuclei of the dividing root cell is now under investigation.

For this study, nucleic acid estimations have been made on nuclei isolated from partially synchronized root meristem cells of *Vicia faba*. In addition, attempts have been made to separate nuclei from normal roots into fractions, containing different interphase stages, by means of sucrose density gradient centrifugation.

Results and Discussion. – Lateral roots of *Vicia faba*, which had been treated for 12 hr with 4 m*M* 5-aminouracil (5-AU) and then transferred to water, showed a peak of mitosis 12 hr later. This confirmed some earlier results of Jakob and Trosko.[2] Nuclei were isolated 6 hr from the beginning of the recovery period when a high proportion were in the G_2 stage. Further samples were isolated 11 hr later when a high proportion, which had recently undergone mitosis, were in G_1. Because of Mattingly's observation[3] that there are at least two discretely synchronous populations which pass through division at different times in different parts of the root, all observations were restricted to nuclei isolated from apical 1.0-mm segments.

The average amounts of DNA per nucleus in the first (6 hr) samples were found to be higher than the average amounts in nuclei isolated concurrently from the roots of untreated controls. In the second (17 hr) samples, the average amounts of DNA per nucleus were lower than those found in controls. In all cases the differences were consistent with the differences in the distribution of Feulgen values obtained microspectrophotometrically. In experiments where the highest degree of synchrony was attained, it was also possible to show the average RNA contents per nucleus in the first samples to be significantly higher than those in the second, although the increases were smaller than the increases in the amounts of DNA. From pulse-labeling experiments using ^{3}H-adenosine and autoradiography it was possible to demonstrate that RNA synthesis had continued during the 5-AU treatment and throughout the recovery period. The fact that the amount of RNA per nucleus in the G_2 population appears to be higher than that in the G_1 population suggests that there is net RNA synthesis during interphase as well as turnover. However, because of the lack of information relating to the possible effects of 5-AU on the level of nuclear RNA synthesis, confirmation of the above results has been sought through the use of a second technique.

Nuclei isolated from normal untreated roots have been fractionated by sucrose density gradient centrifugation using a modification of the method proposed by Fisher *et al.*[4] Three discrete fractions of nuclei have been obtained, and, from their average DNA contents, it seems probable that the two most rapidly sedimenting fractions consist mainly of G_2 nuclei, whereas the third more slowly sedimenting fraction consists of nuclei which are predominantly G_1. The average amounts of RNA per nucleus in these three fractions show remarkably close correlation with the amounts of DNA, and this provides confirmation of a net increase in nuclear RNA associated with the doubling of DNA during interphase. The precise timing of this net RNA synthesis in relation to DNA synthesis and chromosome replication will depend upon measurements at the single-cell level by the kind of cytochemical techniques which have been developed for animal tissue culture cells by Seed.[5]

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20.6 PHOTOSYNTHESIS

W. A. Arnold
W. F. Bertsch
J. R. Azzi
L. W. Jones
R. M. Pearlstein
J. B. Davidson[1]

We are continuing the studies of the delayed light from green plants. Delayed light still seems to be one of the best ways to learn about the energy conversion steps in photosynthesis.

Bertsch is in England, Hill's Laboratory, Cambridge University, studying the delayed light emitted by chloroplasts.

By using short, low-intensity flashes of exciting light, Jones has found conditions under which delayed light requires two excitations.

We have developed a new procedure that apparently allows us to study the photochemical part of photosynthesis all by itself. The experiment consists of four steps: (1) a suspension of green plants is held in the dark at room temperature for 5 min to "relax"; (2) the sample is frozen while still in the dark (−10 to −200°C); (3) the sample is illuminated while cold (we believe that the photochemical reaction takes place, but that enzyme reactions do not); and (4) the sample is rapidly heated in the dark to 100°C. The light emission from the sample as it is heated gives a measure of the amount of photoreaction that took place.

References

[1]Instrumentation and Controls Division.

Biophysics

Radiation Biophysics (21.1–21.13)		Molecular Biophysics	
M. L. Randolph	J. L. Hosszu	R. B. Setlow[a]	D. P. Allison
J. E. Donnellan	R. S. Stafford	J. R. Einstein[a]	W. L. Carrier
J. W. Longworth[a]	J. L. Otis[b]	Ann B. Jacobson	Peggy P. Hester
R. O. Rahn		R. A. McGrath	Janet R. McGrath[a]
		R. A. Weisberg	R. W. Williams
		B. K. Zimmerman	S. D. Leonard[b]
		L. G. Caro[c]	Sandra Roland[b]
		J. S. Cook	Marid Schnös[d]

[a]Dual assignments.
[b]Student trainee.
[c]Leave of absence to Hammersmith Hospital, London.
[d]Visiting investigator from abroad.

Participation in Cooperative Programs: AEC-NIH Chemical Carcinogenesis (22.11–22.13)

21.1 STUDIES OF PLASTID RIBOSOME RNA

Ann B. Jacobson D. P. Allison

Introduction. – Experiments have been initiated to examine some of the biochemical properties of plastid ribosomes in etiolated maize leaves. These experiments have resulted directly from observations made with the electron microscope which reveal that plastid ribosomes exhibit some distinct staining properties as compared with nonplastid ribosomes. In particular, plastid ribosomes appear conspicuously smaller than nonplastid ribosomes when leaf tissue is fixed with glutaraldehyde. With other fixatives plastid ribosomes can be difficult to demonstrate under circumstances where nonplastid ribosomes are readily visible. Preliminary observations appear to indicate that these differences may reflect simple differences in the plastid RNA itself.

Results. – Plastid RNA can be characterized only if it can be distinguished from other cellular RNA. A number of experiments indicate that this is possible. Proplastids in etiolated leaves are large cytoplasmic organelles which undergo a rapid and well-characterized conversion to chloroplasts when exposed to light. Recent biochemical studies have shown that when ^{32}P is administered to etiolated leaves during the initial stages of illumination (1 to 3 hr), there is a very rapid increase in the rate of RNA synthesis. When cells are fractionated, a major portion of the labeled RNA can be shown to be associated with plastids.[1]

This observation has been checked further on tissue sections using electron microscope autoradiography. Etiolated leaves were treated with ^{3}H-cytidine for 4 hr. Some leaves received this treatment in continuous darkness. The remainder received the isotope during 4 hr of illumination. Radioautographs of these leaves show a definite light-stimulated synthesis of RNA both in the nucleus and cytoplasm of the leaf cells. In the nucleus the label is located primarily in the nucleolus. In the cytoplasm labeled RNA is localized almost exclusively within proplastids (Fig. 21.1.1). Estimates of proplastid number per cell vary, but generally range between 20 and 100. Thus, if labeled RNA is extracted from such tissue, the major portion of the label will be plastid in origin, the nucleolar label having in effect been diluted out.

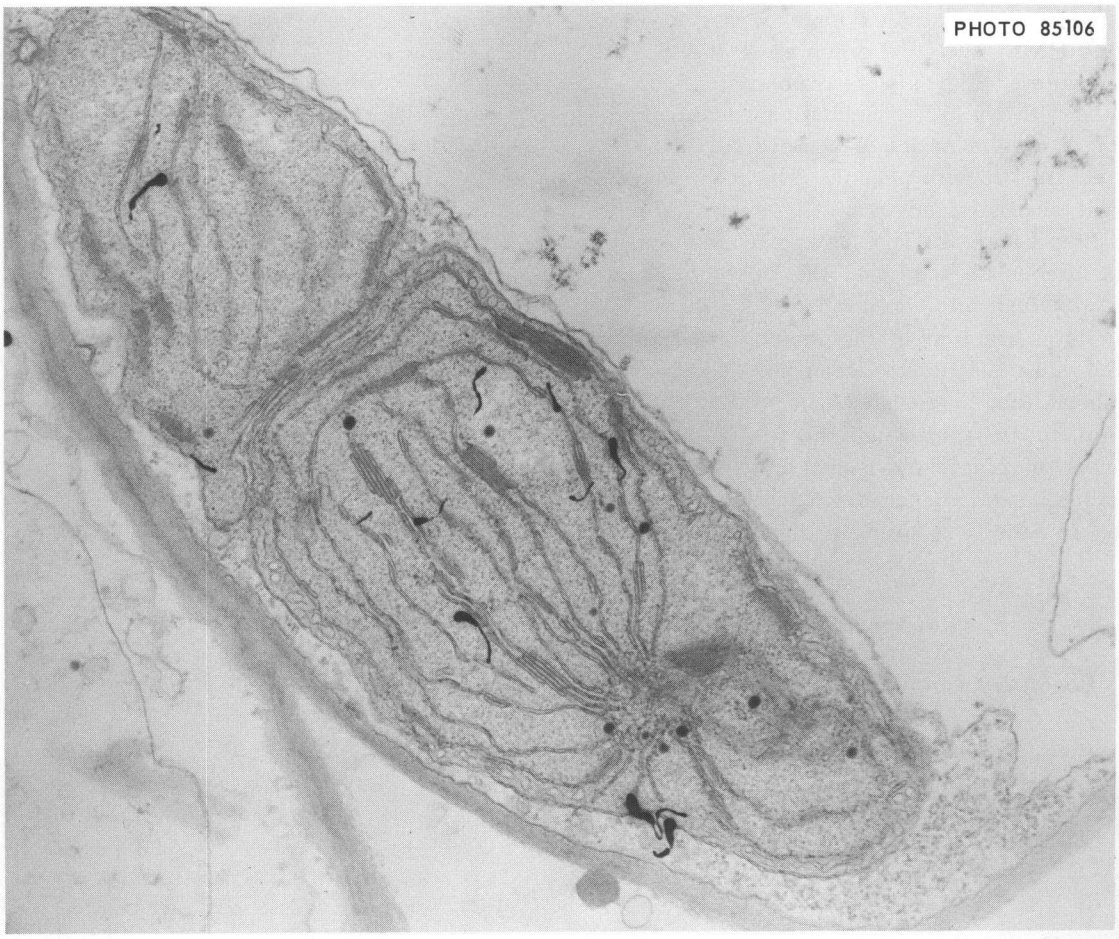

Fig. 21.1.1. Electron Microscope Autoradiograph, Showing Localization of ^{3}H-Cytidine in Proplastids from an Etiolated Maize Leaf Which Had Received 4 hr Illumination. 23,000×.

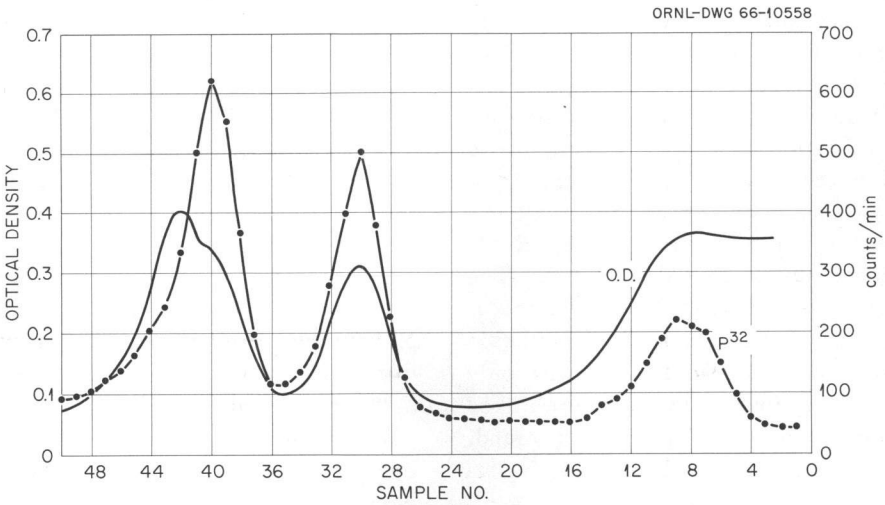

Fig. 21.1.2. Sucrose Density Gradient, Showing Centrifugation Pattern of Ribosomal RNA from Etiolated Maize Leaf Which Had Received 4 hr Illumination in the Presence of ^{32}P.

Some preliminary studies have been made with extracted RNA. A typical preparation is described here. Etiolated leaves were exposed to light and incubated with ^{32}P. The incubation was terminated at 4 hr, a plastid pellet was prepared, and RNA extracted from it with phenol. The RNA was run on 30-ml sucrose gradients (17 to 34% sucrose) for 48 hr. Figure 21.1.2 shows the gradient obtained. It can be seen that the heavy ribosomal peak is resolved into two components. Within this peak a large fraction of the labeled RNA is associated with the lighter component. Since we have shown that labeled RNA is primarily plastid in origin, these results indicate that at least one component of plastid ribosomal RNA is different than cytoplasmic ribosomal RNA. Further investigation is under way to verify this interpretation.

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21.2 THE ENZYMIC SYNTHESIS OF POLYDEOXYNUCLEOTIDES ON dA:dT TEMPLATES

B. K. Zimmerman

The copolymer dA:dT[1] provides a template of defined structure which may be used to determine the mechanism of nascent chain initiation in polynucleotide synthesis by DNA polymerase. In these experiments *Micrococcus lysodeikticus* DNA polymerase was employed. dA:dT will prime for the synthesis of poly dA or poly dT alone as well as for dA:dT. In this way one can study the factors controlling the synthesis of each individual chain in the two-stranded dA:dT duplex.

When dATP is the only substrate used, the dT strand of the primer may be copied only once. The new poly dA strand is covalently linked to the 3′ end of the dT strand of the primer. The synthesis of poly dT alone proceeds very slowly, with only about 10% of the newly synthesized strands linked covalently to the 3′ end of the primer dA strand. The low rate of synthesis of poly dT may be attributed to the fact that in the presence of Mg^{2+}, dA:dT plus an excess of poly dT forms a very stable three-stranded complex. This complex is a very poor primer for poly dT synthesis as shown by priming experiments with the physical hybrid $dA:dT_2$.

In the case where poly dA and poly dT are synthesized simultaneously, the synthesis of both occurs at a substantial rate, with the rate of poly dA synthesis generally greater than that of dT synthesis. dA:dT polymers may be synthesized with molecular weights greater than 10^7, starting with primers of the order of 100 nucleotide pairs long. The ratio of -TpA- pairs to total deoxyadenylate incorporated remains constant at about 0.01, consistent with a model in which alternating blocks of poly dA and poly dT of ~100 nucleotides comprise the chains of the copolymer.

References

[1]The author is grateful to F. J. Bollum, Department of Biochemistry, University of Kentucky Medical Center, Lexington, Ky., for gifts of poly dA, poly dT, and dA:dT.

21.3 EFFECT OF SOME CHROMOSOME-BREAKING AGENTS ON THE DNA POLYMERASE AND NUCLEASE ACTIVITY OF CHINESE HAMSTER CELLS

B. K. Zimmerman J. E. Trosko[1]
J. G. Brewen[2]

A recent theory of Ahnstrom and Natarajan[3] proposed that chemical agents, which induce chromosome breaks, mediate breaks by creating conditions in the cell which favor pyrophosphorolysis of DNA.

Chinese hamster cells were treated with cytosine arabinoside, 5-fluorodeoxyuridine, and excess thymidine at times and concentrations known to cause many chromosome breaks in these cells. Extracts of treated and nontreated cells were obtained by freeze-thawing and assayed for DNA polymerase, endo-, and exonuclease activity. No difference in DNA polymerase or exonuclease activity was found for any of the treated cells when compared to the control. The exonuclease enzyme greatly preferred single-stranded DNA over native DNA. No endonuclease activity was detected under conditions used in this survey in the Chinese hamster line.

We can only conclude that, at least on the whole cell extract level, these chromosome breaking agents do not significantly affect the enzymic reactions analyzed in this study.

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21.4 THE CONTINUITY OF *ESCHERICHIA COLI* DNA

R. A. McGrath R. W. Williams

By lysis of cells and centrifugation of their DNA in alkaline sucrose gradients, we showed that radiation-resistant bacteria (such as *E. coli* B/r) can rejoin pieces of DNA broken by x irradiation. Radiation-sensitive bacteria such as B_{s-1} do not rejoin broken DNA.[1] The extension of these studies to higher organisms requires the isolation of DNA from cells with a minimum number of breaks produced by mechanical manipulation. While developing further modifications of the methods, we discovered preliminary evidence that the *E. coli* chromosome may consist of six segments.

PPLO (pleuropneumonia-like organism) strain *M. laidlaw B* was obtained from Mark Tourtellotte of the University of Connecticut. The DNA of this organism is circular, with a molecular weight of 4×10^8 daltons per single strand. This value is twice the molecular weight of the pieces of *E. coli* DNA that can be isolated by our technique ($\frac{1}{6}$ the single-strand molecular weight). We were able to compare the sedimentation profiles of *E. coli* and PPLO DNA. The following facts were found: (1) PPLO DNA is very sensitive to shear, but pieces can be isolated with a molecular weight between 2 and 4×10^8 daltons; (2) the shape of the sedimentation profile for *E. coli* has a sharp leading (high molecular weight) edge, but that for PPLO has a nearly symmetrical distribution. The sedimentation density profile for *H. influenzae* (molecular weight $\sim 2 \times 10^8$ daltons) is also symmetrical.

Although DNA from PPLO or *E. coli* may be sheared during lysis and centrifugation, the peculiar shape of the *E. coli* sedimentation profile can be interpreted as follows: The majority of DNA molecules in this organism are in segments of lengths about 2×10^8 daltons. Pieces of this length are not broken during manipulation. Thus the chromosome of *E. coli* is composed of six linear pieces of DNA joined in end-to-end fashion by alkali-labile bonds. The symmetrical distribution from the other organisms studied favors the interpretation that in these cells DNA is longer than 2×10^8 daltons and such pieces are randomly broken during lysis and centrifugation.

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21.5 PHOTOREACTIVATING ENZYME IN ANIMALS

J. S. Cook Jane K. Setlow[1]

Introduction. – Photoreactivation of uv damage is a widespread biological phenomenon, having been demonstrated in many plant and animal phyla. There is considerable evidence that the mechanism of one type of photoreactivation is the splitting of uv-induced pyrimidine dimers in DNA by a photoreactivating enzyme in the presence of light. However, in some organisms photoreactivation is indirect and does not involve either a dimer-splitting reaction or a light-dependent enzyme which acts directly on DNA. Thus, the demonstration of photoreactivation in an organism does not necessarily imply the existence of a photoreactivating enzyme. Up to the present report *Neurospora* is the most highly developed organism which has been shown to contain such enzyme. We have tested extracts of the sea urchin *Arbacia punctulata*, rat liver and testes, rabbit skin, and mouse ear for photoreactivating activity.

Results and Discussion. – None of the mammalian extracts showed any evidence of photoreactivating activity. However, extracts of sea urchin eggs or testes have been shown to contain enzyme by the following three criteria: (1) the ability of the extracts to photoreactivate uv-inactivated transforming DNA, (2) the destruction of this photoreactivating ability by proteolytic enzymes and heat, and (3) the ability of the extracts

to split uv-induced pyrimidine dimers in purified *Escherichia coli* DNA.

Cleavage delay induced by uv in *Arbacia* eggs is photoreactivable. This fact, together with our results, suggests that most or all of the photoreactivable lesions which cause this cleavage delay are pyrimidine dimers.

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21.6 STRAND BREAKS THAT RESULT FROM THE EXCISION OF PYRIMIDINE DIMERS FROM DNA

R. B. Setlow W. L. Carrier
R. W. Williams

Introduction. – Bacterial strains that are resistant to the effects of uv radiation are able to excise intrastrand pyrimidine dimers from their DNA. The dimers are removed as parts of oligonucleotides, and thus excision results in the formation of single-strand breaks in the bacterial DNA. The time devoted to the associated steps in repair of DNA – repair replication and the rejoining of strands – can determine whether repair is successful and a cell survives. We have measured the numbers of strand breaks in bacterial DNA during excision in vivo.

Methods. – Cells of *E. coli* B, labeled with ^{3}H-thymidine, were irradiated with 200 ergs/mm^2 of 265-mμ radiation – a dose that makes about 500 pyrimidine dimers per single strand of the bacterial chromosome. The cells were placed in growth medium after irradiation, and fractions of the culture were analyzed at various times for dimers remaining in DNA and for the numbers of single-strand breaks. The latter measurement was made using the technique of McGrath and Williams[1] for the extraction and analysis of large pieces of single-stranded DNA from bacteria. The DNA extracted from unirradiated cells contains about six breaks per strand.

Results. – During the time that dimer excision is taking place there is an increase and then a decrease in the number of single-strand breaks in DNA (Fig. 21.6.1). By the time excision is complete the DNA extracted from irradiated cells is like that of unirradiated cells. The efficiency of

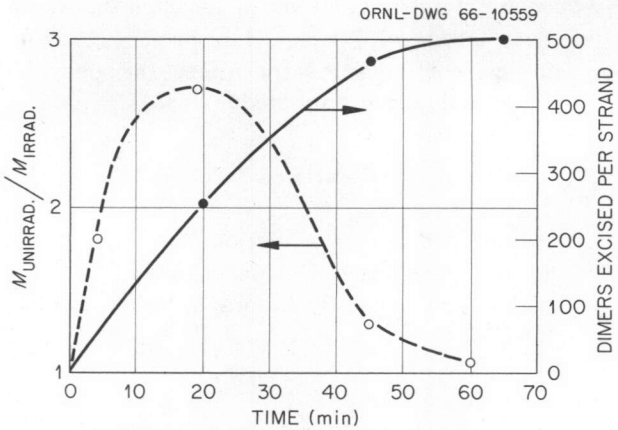

Fig. 21.6.1. Single-Strand Breaks Found During Excision of Dimers in *E. coli* B. The number of breaks is approximately equal to the ratio of the molecular weight of unirradiated to irradiated DNA multiplied by 6, the number of breaks observed in the extraction of unirradiated cells.

the uv-repair process is indicated by the small numbers of single-strand breaks that exist while dimers are being excised. Even though 15 dimers per minute are being excised for about 30 min, there are only approximately 15 strand breaks at any instant. The strand breaks are repaired almost as soon as they are made.

There is a big difference between the repair of x-ray-induced breaks and breaks that arise as a result of excision of dimers. In 60 min only 20 x-ray breaks are repaired,[1] but 500 excision breaks per strand are rejoined. The two types of breaks are not only of different lengths but probably have different kinds of end groups.

References

[1]R. A. McGrath and R. W. Williams, *Biol. Div. Semiann. Progr. Rept. July 31, 1965,* ORNL-3853, p. 158.

21.7 LOSS OF PYRIMIDINE DIMERS FROM BACTERIAL DNA AS A RESULT OF PHAGE INFECTION

R. B. Setlow W. L. Carrier

Introduction. – Ultraviolet irradiation of extracellular T4 bacteriophage forms thymine dimers

in the phage DNA. When such phage infect cells of *E. coli* the dimers in the phage DNA are excised if the phage carry the v gene.[1] There is no excision if the phage are v^-, even though the bacterial cells contain enzymes that excise dimers from bacterial DNA. Since the bacterial system does not work on dimers in phage DNA, we looked to see if the phage system works on dimers in bacterial DNA.

Method. – Cells of *E. coli* B_{s-1} (a strain unable to excise dimers from its own DNA) were labeled with ^{3}H-thymidine and irradiated with 100 ergs/mm^2 of 265-mμ radiation. They were infected with an average of 5 phage per cell and at 20 min after infection were analyzed for thymine-containing dimers in the TCA-insoluble fraction.

Results. – Irradiated cells contained 0.080% of their thymine radioactivity in dimers. After infection with T4 v^- and T4 v^+ phages there was 0.059 and 0.028% radioactivity in dimers respectively. We interpret these and similar experiments as follows. Infection with either type of phage results in some breakdown of the bacterial DNA to mononucleotides and dimer-containing oligonucleotides. The mononucleotides are incorporated into phage DNA, but the dimers are not, and hence the fraction of dimers in the TCA-insoluble fraction decreases even though no excision has taken place. In addition infection with T4 v^+ results in a further loss in dimers from DNA – a loss that probably represents excision of dimer-containing oligonucleotides as a result of enzymic action under the control of the v gene of the phage.

References

[1]R. B. Setlow and W. L. Carrier, *Biol. Div. Semiann. Progr. Rept. July 31, 1965,* ORNL-3853, p. 159.

21.8 EXCISION OF DIMERS IN VITRO

W. L. Carrier R. B. Setlow

Extracts of *Micrococcus lysodeikticus* are able to excise pyrimidine dimers from uv-irradiated DNA[1] and thymine dimers from irradiated dA : dT. The excision is accompanied by little hydrolysis of the polynucleotides. The in vitro excision process is presumed to be similar to those observed in vivo in radiation-resistant cells.

The isolation and purification of the enzyme(s) are in progress. The dimer excision activity of the extract does not stick to DEAE-cellulose, but it does interact with DEAE which has been saturated with DNA (a technique devised by Zimmerman and Howard).[2] Chromatography on DEAE-DNA has yielded a protein fraction with a much higher specific activity than the crude extracts.

References

[1]W. L. Carrier and R. B. Setlow, *Biochim. Biophys. Acta,* in press.

[2]B. K. Zimmerman and Patricia N. Howard, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1966,* ORNL-3922, p. 157.

21.9 EFFECT OF UV IRRADIATION ON SPORE DNA

J. E. Donnellan R. S. Stafford

Acid hydrolysis of uv-irradiated bacterial spores yields thymine-containing photoproducts that are different from the pyrimidine dimers produced in vegetative cells. The presence of the photoproducts in spore DNA is correlated with survival of bacterial spores, because spores produced in a medium containing added $MnSO_4$ were 2.3 times more resistant to uv irradiation, while their DNA contained 2.4 times fewer thymine photoproducts than spores produced in medium without added $MnSO_4$.[1] In order to characterize the spore photoproducts they were produced in dried DNA and in dried thymine, thymidine, thymidylic acid, and synthetic polynucleotides.

Results and Discussion. – DNA was extracted from *Escherichia coli* grown with thymidine labeled with ^{14}C in the 2 position or tritium in the methyl group or in the 6 position. The labeled DNA, in Tris buffer, was dried with an equal weight of calcium dipicolinate and irradiated at a wavelength of 2537 A with approximately 2×10^5 ergs/mm^2. The amounts of photoproducts produced were determined following acid hydrolysis and separation of photoproducts by paper chromatography. The same quantities of photoproducts, having properties identical to the spore photoproducts, were produced in DNA's having the radioactive label in different positions. Traces (2 to 3%) of

the radioactive thymine were converted to photoproducts behaving chromatographically like normal pyrimidine dimers (UT and TT). Drying the DNA in the presence of NaCl resulted in a decrease in the amount of spore photoproduct and an increase in the photoproduct in the dimer region. The irradiation of the synthetic polynucleotide dA:dT resulted in a greater amount of thymine conversion into photoproducts of all types, whereas irradiation of the alternating polynucleotide dAT resulted in a reduced conversion. Irradiation of DNA extracted from T4 phage and dried in the presence of calcium dipicolinate yielded normal amounts of photoproducts, indicating glucosylation did not affect photoproduct production. Only small amounts of photoproducts were produced when the dinucleotide TpT was irradiated dry.

Irradiation of dried thymine, thymidine, or thymidylic acid resulted in the conversion of up to 6% of the radioactivity into photoproducts having chromatographic properties similar to the spore photoproducts. Chromatography of irradiated dried TMP (not hydrolyzed) on DEAE paper gave products with mobilities expected for dimers.

Conclusion. – These studies indicate that the conformation change which occurs upon drying DNA results in an inhibition of normal pyrimidine dimer production and a large enhancement of the production of different photoproducts. The new photoproducts appear to be dimers of thymine with little change in the basic thymine structure. Experiments are in progress to isolate large quantities of the photoproducts from irradiated dried thymidylic acid and thus characterize its structure.

References

[1]J. E. Donnellan, Jr., and R. S. Stafford, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1966*, ORNL-3922, p. 148.

21.10 SHORT WAVELENGTH REVERSAL OF URACIL DIMERS IN POLY U AT 77°K

R. O. Rahn J. W. Longworth

Introduction. – It is known that photoinduced dimer formation in poly U is strongly inhibited at 77°K.[1] Experiments were carried out to determine whether short wavelength reversal of the dimers was also inhibited in the frozen state.

Methods. – Poly U was dissolved in an ethylene glycol and water mixture which forms a clear glass upon freezing. Irradiation was first carried out at room temperature with 265-nm radiation to give a final relative absorbance of 0.32 at 260 nm. Short wavelength reversal was then carried out at both 298°K and 77°K with 238-nm radiation.

Results. – As shown in Fig. 21.10.1 the maximum amount of reversal, without intermittent thawing and refreezing, was about four times less at 77°K than at 298°K. When a single sample was repeatedly irradiated at 77°K, thawed, and reirradiated at 77°K, then the maximum amount of reversal was at least equivalent to that found at room temperature (see curve labeled "interrupted" in the figure). In all cases the initial rate of reversal was the same.

It is clear that irradiation at 77°K inhibits both the formation and the splitting of uracil dimers in poly U. However, the inhibition of reversal as shown here manifests itself not by a change in the initial rate of reaction but only by a change in the final equilibrium distribution. Such a situation is not consistent with either a change in the rate constants k_1 and k_2 defined by

$$\mathrm{UpUp} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \widehat{\mathrm{UpUp}}$$

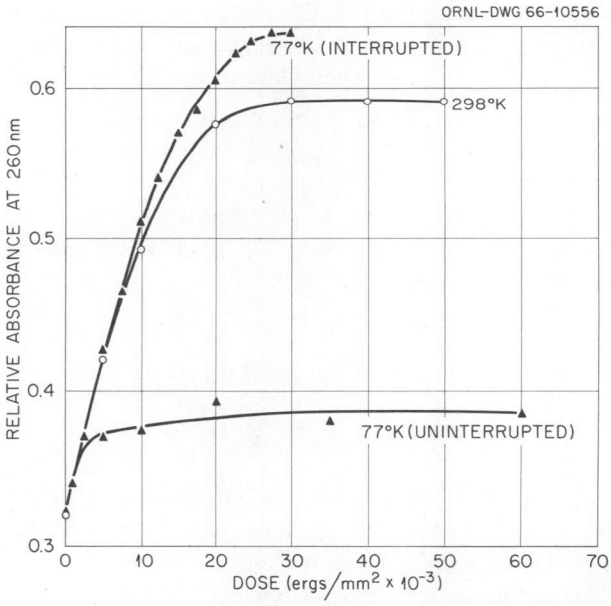

Fig. 21.10.1. Reversal of Uracil Dimers in Poly U with 238 nm Irradiation at Room Temperature (298°K) and at 77°K.

or a decrease in the concentration of dimers capable of being split at 77°K. In either case the initial rate of reaction would be smaller at 77°K than at room temperature, and this was not observed. A model consistent with the observations is one in which the quantum yield of reversal at 77°K is the same as at room temperature for all the dimers until a certain portion (25%) are split. Then the quantum yield for reversal falls to zero. Thawing and refreezing restores the initial quantum yield.

References

[1]R. O. Rahn, submitted to *Science*, 1966.

21.11 ELECTRON SPIN RESONANCE OBSERVATIONS OF THE TRIPLET STATE IN TYROSINE AND POLY-L-TYROSINE

R. O. Rahn J. W. Longworth

It is easy to distinguish between the triplet resonances of neutral and ionized tyrosine (pH > 10). The peaks of their resonances are separated by approximately 80 gauss; furthermore, it is possible preferentially to excite the ionized tyrosine by means of 305-nm radiation, whereas 285 nm excites both neutral and ionized molecules about equally.

Both tyrosine and poly-L-tyrosine were made up 0.2 mg/ml in a 5:4:1 ethylene glycol:dimethyl sulfoxide:water solvent. The state of ionization was varied by adding NaOH. Low-temperature absorbance measurements were made to determine the state of ionization at 77°K – the temperature at which the ESR measurements were made.

The intensities of the triplet ESR signals for neutral and ionized tyrosine for excitation with 285 nm were proportional to the amount of each species present and varied linearly with the degree of ionization. A linear dependence on concentration was also obtained for the ionized signal with excitation by 305 nm. The linear dependence of the ionized signal for excitation with either wavelength means that the ionized triplet is populated directly by means of ionized absorption and that neutral absorption makes no contribution to the ionized triplet.

A different situation was found in polytyrosine. Because of the proximity of the tyrosyl groups in the polymer, energy transfer processes are more highly favored and have been observed in polytyrosine by means of fluorescence techniques. Singlet energy transfer was shown to take place from the neutral to the ionized molecule (this report, Sect. 22.13). Excitation with 285 nm can excite ionized tyrosine not only directly but also indirectly by means of neutral absorption followed by energy transfer. The extent of energy transfer contributing to the observed ionized signal in polytyrosine was found for various states of ionization by measuring the signal heights for 285-nm and 305-nm excitation. The amount of energy transfer in polytyrosine falls off rapidly as the number of ionized groups increases. There is no comparable transfer in tyrosine.

21.12 MOISTURE DEPENDENCE OF ENDOGENOUS ESR SIGNALS IN SEEDS

M. L. Randolph John A. Heddle[1]
J. L. Hosszu

Introduction. – Previously we reported an increased ESR signal in seeds, especially all varieties of onion, on heating the seeds for various durations.[2] In order to understand this phenomenon better we have measured ESR signal heights vs a measure of water content of seeds.

Results. – The water content of seeds of *Allium cepa,* the white onion, was varied by storing seeds for predetermined durations in desiccators containing various drying agents – 50°C air, 80°C air, $CaCl_2$, H_2SO_4, high vacuum at room temperature, and house vacuum at 80°C – and saturated air at room temperature as a moistening technique. Thereafter the seeds were divided into two groups, one of which was used for moisture determination by change of weight during a standardized heat treatment, and the other group used for measurement of ESR signal height. As shown in Fig. 21.12.1, an inverse relationship between ESR signal and moisture was found regardless of the treatment used to change water content. The continuity of results for samples which were first dried and then remoistened suggests that the effect is reversible. A purely empirical fit to the data is shown in the figure.

Discussion and Conclusion. – Apparently the ESR signal height of endogenous signals in these onion seeds is reversibly related to this measure of water content. Although the mechanism of the phenomenon is unproven, it may simply be the

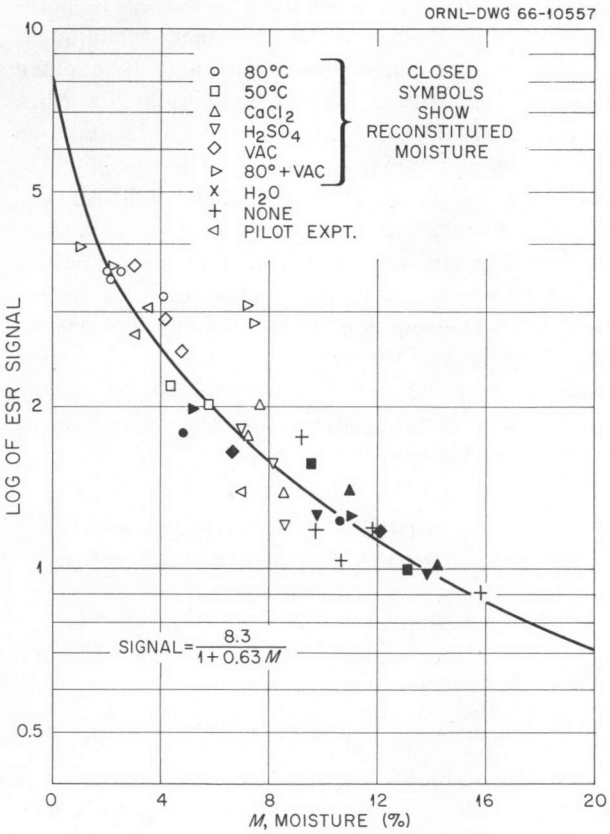

Fig. 21.12.1. Dependence of ESR Signal on Moisture Content of Onion Seeds.

result of a change of the equilibrium constant between two valence states as the water content varies. In any event, this study indicates that water content of seeds may significantly change the amplitude of ESR signals which seeds exhibit without other treatment.

References

[1]Present address: Radiobiological Research Unit, Medical Research Council, Harwell, England.

[2]M. L. Randolph, J. A. Heddle, and A. H. Haber, *Biol. Div. Semiann. Progr. Rept. July 31, 1965,* ORNL-3853, pp. 155–56.

21.13 RECALIBRATION OF CHRONIC ^{60}Co GAMMA-RAY FACILITY

M. L. Randolph Marvin Slater[1]

Introduction. – This work completes the modernization and reappraisal of the dosimetry for the chronic fast-neutron and gamma-ray dosimetry facility established ten years ago for studies of delayed mammalian RBE effects at various dose rates. The neutron reappraisal has been reported previously.[2]

Results. – The bulk of the results were obtained from evaluation of ionization measurements made in 1955 and 1959 with polyethylene (PE), polystyrene (PS), and Victoreen chambers. The latest accepted constants – such as relative stopping powers, mass-energy absorption coefficients, specific emission, and conversion from roentgens to rads – were used throughout.[3] The various results of the independent or semi-independent measurements are given in Table 21.13.1. The maximum difference between measurements is about 12%; and the width of the 95% confidence interval for the average of all values treated as independent is about ±2% of the average. The reappraisal for dose rate near the source does not differ significantly from the initial value, nor do the two sets of data differ significantly. However, at distances greater than 1 m from the source, dose rates higher than predicted from the inverse square relationship were observed, presumably caused by scattering of gamma rays. Mayneord and Roberts[4] have suggested the scattering correction for a source at the center of a spherical room, and Hendee and Ellis[5] have measured the correction for sources over an infinite plane. Our physical situation lay between these extremes, and hence the scatter data at great distances was empirically fitted to a formula having a term for each model.

Conclusion. – The accuracy of the basic dosimetry for thin biological materials seems to have been well within ±5% except at the greater distances, where, because of the uncertainty in the scatter estimate, the limits may be ±10%.

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[1]Deceased.

[2]M. L. Randolph and Marvin Slater, *Biol. Div. Semiann. Progr. Rept. Jan. 31, 1966,* ORNL-3922, pp. 150–51.

[3]*Physical Aspects of Irradiation,* Natl. Bur. Std. (U.S.) Handbook 85 (1964).

[4]W. V. Mayneord and J. E. Roberts, *Brit. J. Radiol.* **10**, 365–88 (1937).

[5]W. R. Hendee and B. L. Ellis, *Health Phys.* **12**, 673–81 (1966).

Table 21.13.1. Intercomparison of Dosimetry Methods for Chronic ^{60}Co Gamma-Ray Facility

Dosimetric Method	Ion Chamber	Dose Rate in Muscle Corrected for Decay (rads/hr at 1 cm)	
		1955	1959
Absolute ion chamber	PE	1642	1749
	PS	1710	1790
	0.25 r Victoreen		1829
Calibration vs Ra	PE	1680	1710
	PS	1692	1710
	0.25 r Victoreen		1802
Indirect use of NBS calibration vs ^{60}Co	25 r Victoreen	1790	
ORNL isotopes		1761	
Average (95% CI)		1712 ± 57	1765 ± 53
Overall average			1739 ± 36
Value from initial appraisal of 1955 data		1710	

Cooperative Programs

AEC-NIH CHEMICAL CARCINOGENESIS

Biochemistry of Carcinogenesis (22.1)

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B. L. Cain
W. L. Fox
Mary L. Hummel
D. W. Parsons
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Enzymology of the Carcinogenic State (22.2–22.5)

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Beryl Ortwerth[b,c]
L. C. Waters[b,c]
Kiki B. Hellman[b]
E. L. Murray[b]
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Mammalian Chemical Carcinogenesis (22.6, 22.7)

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Chromosomal Effects of Chemicals and Radiation (22.8)

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Effects of Radiation and Chemicals on Paramecium (22.9, 22.10)

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Biophysics of Carcinogenesis (22.11–22.13)

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J. R. Einstein[b]
J. W. Longworth[b]
C. H. Wei
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Chemical Mutagenesis in Microorganisms (22.14, 22.15)

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Inhalation Carcinogenesis (22.16–22.22)

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A. P. Harrison, Jr.
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Alfred Hellman[f]
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T. E. Banker[g]

[e]Leave of absence to Karolinska Institute, Stockholm.
[f]Loanee.
[g]Temporary.

22.1 STUDIES OF THE MECHANISM OF ACTION OF ONCOGENIC VIRUSES

Stanfield Rogers J. H. Evans

In this past interval, because of a turnover and shortage of technical personnel, work has been primarily limited to studies of the nuclear histones in the Shope virus-induced papillomas. We previously reported that upon comparing the histone content histochemically, there is a reduction in the arginine-rich histones in the papilloma as compared to normal or hyperplastic skin.[1] Upon electrophoretic separation of the nuclear histones of papilloma, one histone band found in normal or hyperplastic skin is missing in the papilloma. This missing band appears in the papillomas of rabbits following parenteral injection of arginine and canavanine to bypass the virus-induced arginase. From previous work such treatment is known to stop the growth of the papillomas.[2]

When the bands found in normal skin are stained with Fast Green, a procedure known to be dependent upon the amount of arginine in the histones, this band missing in the papilloma, together with another faster moving band, stains. The third major band of skin histone does not stain. The amount of arginine found in the histones derived from the papillomas induced with the virus is significantly less than that found in normal skin histone. The isolation of the bands and their amino acid analysis are under way but as yet incomplete. The arginine content of proteins of the papilloma, other than the nuclear histones, is relatively the same as in normal skin. These results are consistent with our hypothesis that the action of the virus-induced arginase is to deplete the pool of arginine available for nuclear histone synthesis, thereby depressing the DNA of the cells and resulting in faster growth and the existence of the papilloma. It is conceivable that the histone missing in the papilloma, as manifest by the absence of one band found in normal skin, is the histone controlling the part of the rabbit skin genome specifically related to growth.

Efforts to Selectively Modify the Genetic Information Carried by Viruses. – This work is just beginning. C. A. Knight of the Virus Laboratory of the University of California has supplied us with tobacco mosaic virus RNA, to which we hope to add polyuridine using polynucleotide phosphorylase. The Shope papilloma virus DNA is a continuous helix, and prior efforts done in conjunction with Fred Bollum to add polynucleotides failed, presumably because of the closed helix. Adeno-12 virus DNA, in contrast, is a linear helix, though the DNA is noninfective, possibly because the infective form is really continuous like the papilloma virus. Maurice Green of St. Louis University School of Medicine has supplied us with adeno-12 and adeno-2 virus DNA. We are testing whether polynucleotides may be added using Bollum's end terminal addition enzyme. It is also hoped that the enzyme will tie the ends of the linear helix together and possibly make the DNA infective.

Miscellaneous Problems. – Using glycerine gradients, it has been found possible to purify the domestic-rabbit recoverable line of the Shope virus. Interestingly, it bands in a different position than the wild type, moving considerably more slowly. This virus variant is now to be characterized and compared to the wild type.

Collaborative studies being done in conjunction with John Trentin of the Baylor University School of Medicine on the mechanism of action of the adeno-12 virus in cultured cells have yielded a marked difference in the way leucine and isoleucine are handled in the virus-infected cells as opposed to the normal counterparts. Efforts are being made to locate the cause of this metabolic defect induced by the virus.

Studies are continuing on the influence of various chemical treatments upon the stability of the structure of liver and virus-induced arginase using optical rotatory dispersion and studies of enzyme activity.

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22.2 DEVELOPMENT OF TISSUE CULTURE LINES FOR STUDIES ON CHEMICAL CARCINOGENESIS

Kiki B. Hellman G. David Novelli

Introduction. – Recent investigations in cell culture have established the feasibility of propagating several mammalian cell lines as discrete units in agitated, fluid suspension. A variety of techniques and environmental conditions have been proposed for the cultivation of tissue cells in the submerged state. We have utilized the rotary shaker method of Earle *et al.*[1] for the continued propagation of the L-M cell in a chemically defined medium and are currently studying methods of scaling up the production of cells so that greater quantities of material can be obtained for subsequent biochemical studies.

Results and Discussion. – The L-M cell is routinely propagated in volumes of 200 ml of medium contained in a 1-liter Erlenmeyer flask agitated at 100 rpm on a New Brunswick rotary shaker and incubated at 34.5 ± 0.5°C. Results of numerous studies in the pattern of growth under these conditions show that the cultures have a mean generation time of approximately 44 hr and a maximum cell density at five to seven days of 1.0 to 1.2×10^6 cells/ml, when initial inocula of 2.0 to 3.0×10^5 cells/ml are utilized. However, yields as great as 1.4×10^6 cells/ml have been attained. Cell viability, as determined by the erythrosin B dye exclusion technique,[2] is maintained between 100 and 98% throughout the seven-day growth cycle. Cultures have been harvested routinely at four days when cells are in the log phase of growth; these cells, when planted in fresh medium, continue growing at a logarithmic rate. Harvests of four- to five-day-old cultures yield between 0.5 and 0.6 g of material per 200 ml volume. Approximately 15.0 g of material has been collected in this manner and frozen at −60°C for future determinations.

Since obtaining cell material in this manner is both tedious and time consuming, other methods are currently being investigated for the propagation of cells in volumes ranging from 5 to 10 liters. One method involves growing cells in a 15-liter New Brunswick fermentor which has been modified to approximate the rotary action obtained when propagating cells in Erlenmeyer flasks on a shaker. The other method consists of the propagation of cells in 10-liter volumes in the 40-liter swirl jug described by Hellman;[3] an initial experiment has resulted in a yield of 0.22 g per 200 ml of culture, or a total of 11 g. However, the medium employed has been shown to be unsatisfactory, as evidenced by results on stock cultures, and is being replaced by the manufacturer.

Sufficient material has now been collected for the determination of certain basic biochemical parameters such as total RNA, DNA, and protein synthesis and for evaluation of approximate sRNA yield. Once the routine propagation of cells in larger volumes (5 to 10 liters) is established, material will be available for future studies analogous to those being carried out in microbial systems.

While the majority of work to date has dealt with the establishment of routine propagation of cells in large volumes, anticipated research will deal with the effect of chemical carcinogens, 3,4-benzpyrene and dimethylnitrosamine, on L-M cell cultures. The physiological parameters of growth rate, cell viability, cell size distribution, as well as biochemical studies of RNA, DNA, and protein synthesis, will be investigated.

In addition, in vivo studies will be undertaken to study the tumor-producing capacity of both treated and untreated L-M cells inoculated into C3H (Andervont substrain) weanling mice.

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[3]A. Hellman *et al.*, submitted to *Applied Microbiology*.

22.3 ETHIONINE CARCINOGENESIS

Beryl J. Ortwert G. David Novelli

Introduction. – While many investigations have been carried out with the tRNA from bacteria and yeast, little has been done with tRNA from mammalian sources. Studies are currently under way to characterize the tRNA from both normal rat liver and ethionine-induced hepatomas. It is the

aim of these studies to correlate the demonstrated ethylation of tRNA by ethionine with its carcinogenic action.

Results and Discussion. – tRNA has been prepared from rat liver in good yield by the method of Wicks.[1] A preliminary separation of this crude mixture of tRNA's was obtained with reversed phase chromatography on a 0.9 cm × 8 ft column according to the procedure of Kelmers *et al.*[2] Aminoacyl-tRNA synthetases have also been prepared by several different procedures and are sufficiently active to allow the assay of 14 tRNA's. Studies are now being carried out to determine the location of these tRNA's in the reversed phase column eluate. A similar distribution of the tRNA from hepatic tumor tissue should indicate any differences resulting from the action of ethionine.

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22.4 LEUCYL-sRNA: CHROMATOGRAPHIC PROPERTIES FROM NORMAL AND T_2-INFECTED *E. coli* B

L. C. Waters E. L. Murray
G. David Novelli

Introduction. – The comparative study of normal and cancerous trout liver sRNA, as previously proposed,[1] has been curtailed, at least temporarily. Currently we are using the reversed phase chromatographic technique[2] to further investigate a previously reported T_2-dependent modification of *E. coli* B sRNA. Sueoka and Sueoka[3] have reported a study in which *E. coli* B aminoacyl-sRNA was compared, with and without phage infection, using methylated albumin–kieselguhr (MAK) chromatography. Of the 17 amino acids examined, only leucyl-sRNA showed significant differences. The modification appeared to involve an alteration of preexisting species of leucyl-sRNA and was dependent upon injection of phage DNA into the host and upon protein synthesis. The poor resolution afforded by MAK chromatography makes it difficult to ascertain the nature of the modification. The superior resolving power of the reversed phase column makes it an ideal tool for studying the system.

Results and Discussion. – MAK chromatography of precharged *E. coli* B (^{14}C or ^{3}H) leucyl-sRNA gave two peaks of radioactivity with the first peak predominating in the normal system and the second predominating in the T_2-infected system as shown previously.[3] Reversed phase chromatography of *E. coli* B leucyl-sRNA on an 0.8 cm × 8 ft column at pH 4.5, in the presence of EDTA, gives at least four and probably five peaks. Peaks I, II, and III are clearly resolved from each other and from IV and V. Peaks IV and V are incompletely resolved from each other. Peaks I, II, and III are the predominant peaks in normal and 6-min T_2-infected *E. coli* sRNA. However, whereas peak I is the major peak in the normal system, peaks II and III are major in the 6-min T_2-infected system. It is therefore apparent that the modification involves at least three, rather than two, species of leucyl-sRNA. Experiments are currently under way to determine whether these changes are results of de novo sRNA synthesis or modification (such as methylation) of preexisting sRNA.

When *E. coli* B is heavily infected with T_2 (causing lysis inhibition) and sRNA is isolated 2 hr after infection, the resulting leucyl-sRNA is chromatographically distinct from either normal or 6-min T_2-infected sRNA. Like the short-term infection, peaks II and III are larger than peak I; however, peaks IV and V are, under these conditions, now comparable to the other three. This change is even more striking than that between normal and 6-min infected sRNA.

Recent reports suggest that an altered chromatographic behavior for a particular aminoacyl-sRNA can result from altered growth conditions, such as starvation. It is difficult to imagine such a specific modification[3] as observed on T_2 infection to be the result of an altered growth condition (see also Lazzarini and Peterkofsky[4]). Moreover, preliminary chromatographic experiments with tRNA from a methionine-requiring, relaxed strain of *E. coli* showed that leucyl-sRNA from methionine-starved cells differs from that of normal cells. The difference seems to be mainly the appearance of one new peak (methyl deficient species?), and the differences are quite unlike those observed with T_2 infection. We intend to investigate further the possibility of the observed modification being unrelated to T_2 infection.

Current plans involve an extension of these techniques to a variety of systems in an effort to learn more about the chemistry and biochemistry of sRNA and its possible involvement in the regulation of protein synthesis.

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22.5 FURTHER STUDIES ON THE AMINO ACID INCORPORATING SYSTEM FROM TROUT LIVER

Lawrence Rosen Shigemi Iiyama
G. David Novelli

Introduction. – The soluble system, which incorporates amino acids into a hot TCA-insoluble product, has been further investigated. The system, which is insensitive to RNase, is found in the high-speed supernatant fraction, 40,000 × *g* for 200 min (S_{200}) from a trout liver homogenate.[1]

Results and Discussion. – The S_{200} is treated with RNase at 2° and then passed through a Sephadex G-50 column. The excluded protein (G_{50}) is a more satisfactory system for study than the original S_{200}. Both the S_{200} and G_{50} are completely inactivated at 37° in 10 min. For this reason assays are done at 15° and RNase is routinely added to the assay mixture. The present system consists of three or more separate reactions or series of reactions; (1) a lag period during which no incorporation occurs, (2) an incorporation period during which phenylalanine is incorporated into a hot TCA-insoluble product, and (3) the degradative period during which the incorporated phenylalanine is solubilized. Experimental results demonstrate that during the incorporation period the other two reactions also occur. To date it has not been possible to separate the degradative reactions from the other two. Despite this complicating factor, certain information relating to the system has been obtained and is discussed below.

Lag Period (LP). – This period lasts for approximately 30 min at 15°, and in this time phenylalanine is possibly converted into an intermediate form for incorporation into product. ATP is definitely required in this period.

Incorporation Period (IP). – This period is characterized by the rapid incorporation of phenylalanine into product. Despite the known occurrence of the degradative reaction during this period, a net increase in incorporation is usually observed for 180 min or longer when the G_{50} is used. With untreated S_{200}, the IP is 60 min or less. The amount of phenylalanine incorporated is proportional to the protein concentration.

Degradative Period (DP). – By removal of ATP from the reaction system during the IP (by the addition of glucose and hexokinase) the lability of product is readily apparent. At 15° the product formed prior to ATP removal has a half-life of 40 min or less.

Although some of the components necessary for the LP and/or IP are completely inactivated at 37° in 10 min, the degradative system is much less heat labile. Thus it has been possible to obtain a dialyzed ammonium sulfate fraction which rapidly degrades the product. The ammonium sulfate fractions, singly or in combination, are incapable of incorporating phenylalanine into product. Although the product is subject to continual degradation, it has been isolated from a reaction system during the IP by passage through Sephadex G-50. More than 90% of the incorporated ^{14}C phenylalanine is found in the excluded protein fraction. No other TCA-precipitable ^{14}C fraction is found in the Sephadex eluate.

Sucrose density gradients show the product to be heterogeneous (approximately 4 to 16*S*). The incorporated phenylalanine is completely solubilized by tryptic digestion.

The above results indicate that phenylalanine is incorporated into a peptide linkage through an unusual route. Due to the unique character of this reaction further studies will be made in order to fractionate the system and to characterize the product.

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Table 22.6.1. Tumors in Mice Ingesting DEN for 22 Weeks

Site	Overall Incidence	Incidence in Histologically Examined Animals	Predominant Type
Lung	24/42 (57%)	20/27 (74%)	Adenoma
Liver	33/42 (79%)	26/29 (90%)	Hepatoma
Forestomach	32/42 (76%)	27/27 (100%)	Squamous cell carcinoma
Esophagus	4/42 (9%)	4/22 (18%)	Squamous cell carcinoma

22.6 DIETHYLNITROSAMINE-INDUCED TUMORS IN RF MICE

N. K. Clapp A. W. Craig[1]
R. E. Toya, Sr.

Introduction. – Diethylnitrosamine (DEN) has been recognized as a potent carcinogen for the production of tumors of the liver and upper respiratory system in experimental animals. The following study was designed to investigate the carcinogenicity of DEN in the RF mouse, an animal particularly susceptible to the induction of leukemia by x rays and radiomimetic chemicals.

Results and Discussion. – Male RF/Un mice were given DEN at a concentration of 4 mg per 100 ml of drinking water for 22 weeks consecutively. Animals surviving the 22-week treatment period consumed approximately 6 mg of DEN per kilogram of body weight daily and received a mean cumulative dose of 943 mg/kg. The mice lost weight from six weeks through the end of the treatment period and failed to regain their initial weights prior to death. The first mouse died in the 12th week of treatment and the last in the 45th week, the mean age of death for all treated animals being 29 weeks, as compared to a normal of 80 weeks for controls. Sixteen animals died or were sacrificed prior to 22 weeks, and no tumors were found. Of mice that died or were sacrificed after 22 weeks, 57% had lung tumors, 79% had liver tumors, and 76% had tumors of the forestomach that could be detected grossly (Table 22.6.1). Based on histologic examination, however, incidence was even higher, that is, 74, 90, and 100% respectively. In addition, microscopic squamous cell carcinomas were found in the esophagus in four animals. Only two cases of leukemia were noted, both lymphatic, indicating that DEN did not exert a leukemogenic effect in this study.

The incidence and types of neoplasms observed in the mice of this study differ in certain respects from those reported heretofore in animals of other species and strains; the reasons for these differences must yet be determined.

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22.7 ESTIMATION OF WATER CONSUMPTION BY INDIVIDUAL MICE CAGED IN GROUPS

N. K. Clapp R. E. Toya, Sr.
A. W. Craig[1]

Introduction. – The administration of carcinogens in the drinking water to mice caged in groups involves the possibility that water intake is not uniform from mouse to mouse. In order to study this possibility, we gave ^{86}Rb, an alkali metal resembling potassium in cellular distribution, in the drinking water to mice.

Results and Discussion. – Seven male mice from a noninbred subline of the RF/Un strain were treated for one day with 3.56 μc of ^{86}Rb per 100 ml in the drinking water. After the radioactivity of the mice decayed to background levels, the animals were treated similarly for a second one-day period, using 7.62 μc of ^{86}Rb per 100 ml of drinking water. Each animal and all cage bedding were counted in an Armac counter and 410-A Auto-Gamma scintillation detector, and the water

volumes consumed by each animal were estimated accordingly. The mean volume of water consumed per mouse was 5.6 ml, with a maximum range of consumption of ±1.0 ml. With the exception of one animal, the consumption by each mouse was essentially the same in both experiments, those mice consuming larger volumes of water doing so in both cases. From this study, we observed no evidence to suggest that water consumption differed significantly among mice within a cage.

References

[1]Christie Hospital & Holt Radium Institute, Paterson Laboratories, Manchester, England.

22.8 AMINOACYL-tRNA SYNTHETASES FROM NORMAL AND NEOPLASTIC HUMAN CELLS

J. D. Regan J. L. Epler[1]
Alfred Hellman

A previous report[2] described the initiation and early steps of this study.

We have now fractionated human liver tRNA by means of countercurrent distribution and assayed the fractions with HeLa[3] synthetase preparations.

By means of a simplified mass culture technique[4] in which HeLa cells (HCAAT subline[5]) are grown in 40-liter carboys, sufficient cells have been accumulated for quantity isolation and fractionation of tRNA's. HeLa tRNA has thus been isolated and fractionated by hydroxylapatite chromatography. Assays of the resulting fractions with HeLa synthetases are in progress. Further fractionation of HeLa and human liver tRNA's, both by countercurrent distribution and by column chromatography, will be performed.

These studies have been somewhat delayed due to a lack of fresh, normal human liver available locally. However, we will now be able to secure this material, certified histopathologically normal, from the National Cancer Institute, Bethesda, Maryland.

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22.9 *N*-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE AS A MUTAGEN FOR *PARAMECIUM*

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

In extending the work on chemical mutagens to other agents of mutagenic and carcinogenic interest, it was found that this agent is highly mutagenic in completely nontoxic doses. Work has been completed on the dose-response curve, and preliminary information has been obtained on the effect of stage in the cell cycle and on the effect of a modifying agent.

Methods. – Paramecia in a known stage in the cell cycle were exposed for 15 min to various concentrations of the agent dissolved in a buffered salt solution. The paramecia were washed free of the agent, isolated into culture medium, and mutation yield determined as in our past work.

Results. – The dose-response curve is shown in Fig. 22.9.1. Unlike the curve for x rays and for the trifunctional alkylating agent triethylenemelamine (TEM), it is highly nonlinear even in the low dose range.

The maximum mutation yield is obtained by treating about midway in the cell cycle. The yield was less when the treatment was given early in the cycle, and negligibly small when given late in the cycle. This is qualitatively, at least, the same as with x rays, alpha particles, ultraviolet light, TEM, and nitrogen mustard.

Posttreatment with streptomycin decreases the mutation yield appreciably, just as it does with other mutagens that have been tried. However, there is some evidence that this treatment continues to be effective even when more than one cell cycle intervenes between the treatment with nitrosoguanidine and with streptomycin. If this evidence is confirmed, it will be the first instance in our experience of a mutational process that remains modifiable for more than one cell cycle.

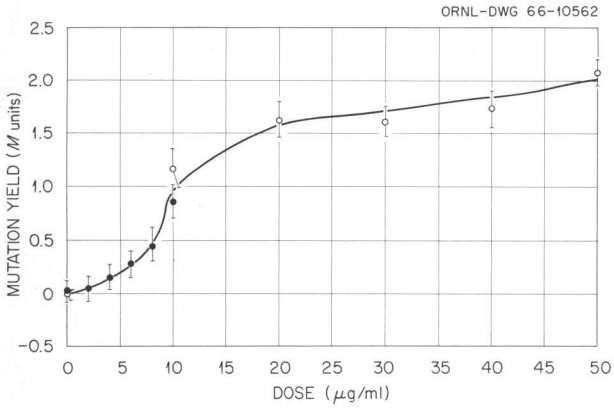

Fig. 22.9.1. Dose Curve for Mutation Induction in *Paramecium* by Nitrosoguanidine. 95% confidence limits shown. Open and closed circles indicate different experiments.

22.10 THE EFFECT OF 3,4-BENZPYRENE ON CELL SIZE AND GROWTH RATE IN *PARAMECIUM*

R. F. Kimball E. G. Bailiff

Our previous work with the hydrocarbon carcinogen 3,4-benzpyrene showed that it gets into the cytoplasm but does not produce mutations nor have any detectable effect on division rate.[1] The only previously detected effect, reported by others as well, was a rapid photodynamic killing of the paramecia in benzpyrene when exposed to a lamp with major output at 3650 A. It seemed a little surprising that there was no detectable effect on such a sensitive parameter as cell multiplication rate when benzpyrene was present in the dark. Therefore, it was decided to check for effects on growth in cell mass as well as on division rate.

Methods. – Benzpyrene (0.1 mg/ml) dissolved in 10% bovine serum albumin and 10% triethylene glycol was obtained through the courtesy of H. V. Malling (Fungal Genetics). Two percent of this mixture was added to culture medium to give a final concentration of 2 μg/ml of benzpyrene. A similar mixture containing all the ingredients except benzpyrene was used for the control. Log phase paramecia in mixed stages were put into these solutions and allowed to grow overnight in the dark. The next morning dividing cells were picked out of both the benzpyrene and control mixtures, allowed to complete division, and one recently divided product was dried on a slide. The other sister was kept in benzpyrene or control medium and used to determine the generation time. These manipulations were carried out in dim yellow illumination. The dry mass of the dried cells was determined individually by microinterferometry.

Results. – In two independent experiments, cells from the benzpyrene cultures were 10% larger than the controls; this difference was significant at the 1% level. There was no significant difference in division rate. There was some evidence that this difference in size may disappear on further culture in benzpyrene. Although these differences are not marked, they do suggest that benzpyrene has a detectable, though not large, effect on cell size. Further studies will be needed to define more clearly the time sequence by which this difference develops. It would appear, however, that the major effect is probably on the rate of growth in mass and not on the rate of cell division.

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22.11 A FORTRAN PROGRAM FOR APPLICATION OF THE MULTIPLE ISOMORPHOUS REPLACEMENT METHOD TO PROTEIN CRYSTAL STRUCTURES

J. R. Einstein

In the method of multiple isomorphous replacement, x-ray diffraction data are collected from an unmodified protein crystal (parent) and from a number of isomorphous crystals containing heavy atoms, which in general are anomalous scatterers. The data do not suffice for unambiguous determinations of the phases of the parent structure factors because of experimental errors and lack of perfect isomorphism. Blow and Crick[1] and others[2] have devised mathematical treatments, the aims of which are to obtain a so-called "best" Fourier series for the electron density in spite of the errors. These treatments have been shown to be somewhat incorrect as applications of probability theory; an alternative treatment has been devised.[3] A FORTRAN program based on the new treatment

has been written for computer calculation of the coefficients for the "best" Fourier. The calculations require only a little more computer time than those based on the older treatments. Several examples have been calculated. The program is applicable to full sets of protein x-ray data.

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22.12 FLUORESCENCE OF POLYTRYPTOPHANS AT ROOM TEMPERATURE

J. W. Longworth R. O. Rahn[1]

A comparison has been made between the fluorescence of poly-L-tryptophan and poly-DL-tryptophan dissolved in the same solvent. The stereoregular polymer poly-L-tryptophan in nonaqueous solvents has most probably the α-helical amide conformation, around which the indolyl groups form another 3.7 helix. Cory-Pauling-Koltun space filling models of the syndiotactic poly-DL-tryptophan do not form a compact helix, but have the least steric repulsions in an extended amide conformation, with the indolyl groups separated by 10 to 12 A and having little regularity.

Poly-L-tryptophan has a fluorescence peak at 333 nm, whereas poly-DL-tryptophan fluoresces at 342 nm, with a 28% greater quantum yield. This shows that the fluorescence of tryptophanyl groups is dependent on their local environment. Similar spectral shifts and quantum yield changes are observed when proteins undergo a conformational change.[2,3]

Both forms of polytryptophans have their neutral fluorescence quenched in alkali, though there is no indication in the absorption spectrum of an ionization at the indole nitrogen. The neutral fluorescence is replaced by a new fluorescence peak at 365 nm. The cause of this new band and the disappearance of the neutral fluorescence is an excited state ionization of the indole nitrogen hydrogen, which is considerably more acidic in the excited singlet state than in the ground state.

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22.13 FLUORESCENCE ENERGY TRANSFER IN POLY-L-TYROSINE AND RELATED MODEL COMPOUNDS

J. W. Longworth R. O. Rahn[1]

The fluorescence of tyrosine compounds at both 77 and 298°K has been studied as a function of ionization of the phenolic hydroxyl groups. Neutral tyrosine compounds have a maximum absorbance at 278 nm and fluoresce in the range 298 to 308 nm depending on temperature and the compound. The ionized forms absorb at 300 nm and fluoresce in the range 330 to 350 nm, and have a yield of 10 to 15% of the neutral fluorescence. The isosbestic point lies at 284 nm, and the identical intersection in the fluorescence – the isoemissive point – lies at 353 nm for 298°K and 345 nm for 77°K. Thus, irradiation at 284 nm excites both neutral and ionized forms proportional to their concentrations, while irradiation at 300 nm excites only the ionized form.

The neutral fluorescence of L-tyrosine decreases proportional to the percentage ionization for 284-nm excitation, while the ionized fluorescence increases in a reciprocal manner for both 284- and 300-nm excitation (Fig. 22.13.1, curves *a* and *b*). (The greater quantum yield for the neutral fluorescence for the initial 15% ionization is due to the ammonium to amino titration of the amino acid.) L-Tyrosine behaved similarly at both 77°K and 298°K, and so has an apparent excited-state ionization constant identical to the ground-state value. In marked contrast is poly-L-tyrosine's neutral fluorescence (Fig. 22.13.1, curve *d*). The quenching constant for the tyrosinate quenching of tyrosine was 0.96 at 77°K and 0.98 at 298°K. The

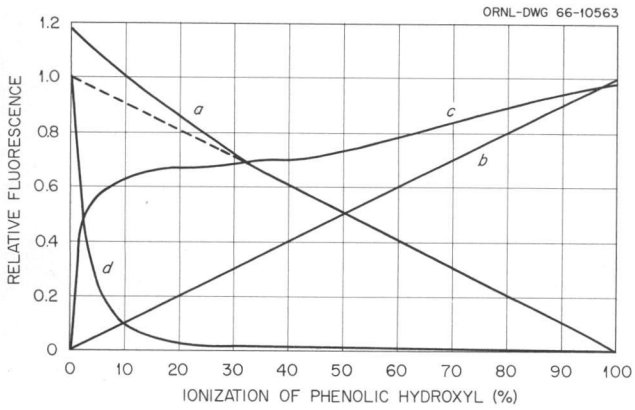

Fig. 22.13.1. Ionization Dependence of Fluorescence. (*a*) L-Tyrosine, neutral fluorescence (305 nm), excitation 284 nm. (*b*) L-Tyrosine, ionized fluorescence (350 nm), excitation 284 and 300 nm; poly-L-tyrosine, fluorescence 350 nm, excitation 300 nm. (*c*) Poly-L-tyrosine, ionized fluorescence 355 nm, excitation 284 nm. (*d*) L-Tyrosine, neutral fluorescence (305 nm), excitation 284 nm.

ionized fluorescence excited at 300 nm behaved like L-tyrosine, that is, was linearly proportional to the percentage ionization (curve c).

Measurements of excitation spectra at the isoemissive point reproduced the absorption spectrum for L-tyrosine L-tyrosinate equilibrium. For poly-L-tyrosine with 10% ionization, the fluorescence at the isoemissive point is largely characteristic of the ionized form, but the excitation spectrum is found to be essentially that of the neutral molecule. Thus there is clear evidence for dipole-dipole resonance energy transfer of tyrosine to tyrosinate in the polymer poly-L-tyrosine.

The related compounds L-tyrosyl-L-tyrosyl diketopiperazine and L-tyrosyl-D-tyrosyl diketopiperazine show similar evidence for tyrosine to tyrosinate energy transfer with quenching constants of 0.90 and 0.76 respectively. These compounds differ only in their conformation, having different separation and orientation of the aromatic rings, so that a measurement of the extent of energy transfer can in principle be related to molecular conformation.

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22.14 *N*-METHYL-*N*′-NITRO-*N*-NITROSOGUANIDINE AS A MUTAGENIC AGENT FOR *NEUROSPORA CRASSA*

H. V. Malling F. J. de Serres

N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) is a potent mutagen in both bacteria[1] and fungi.[2] NTG has also shown potent carcinogenic activity in rodents. At present the experimental evidence favors the concept that the toxicity and carcinogenicity of the *N*-alkyl-*N*-nitroso compounds, for example, NTG, depend on the formation of one or more species of alkylating agents, that is, diazoalkanes, carbonium ions, or both. NTG is most likely to form the methyl derivatives of the alkylating agents. One of those alkylating agents, diazomethane, has been tested for its reactivity with deoxyribonucleosides.[3] Diazomethane reacts readily with guanidine deoxyribonucleoside and thymidine, whereas adenine deoxyribonucleoside and cytosine deoxyribonucleoside are resistant. The basis for this potent mutagenic and carcinogenic activity is unknown. It is possible that NTG is a potent mutagen because it produces a particular type of genetic alteration. To test this possibility we have attempted to characterize the NTG-induced genetic alterations at the molecular level.

In the first series of experiments reversion was studied by treating eight different mutants comprising a tester set.[3] These tests provide a much simpler assay of mutagenicity than most forward-mutation systems, and mutagenicity can be assayed over a wider range of environmental conditions.

The reversion mechanism of the mutants comprising the tester set has been characterized earlier.[4] In the present experiments we found that NTG induces reversions in all mutants which revert by base-pair substitution, with no preference for either base-pair transition (see Table 22.14.1). One of the mutants, 2-17-7, which reverts by base-pair insertion or deletion, may also revert after treatment with NTG. However, more experiments are needed to determine the significance of this low level of reversion.

The characterization of the mutagenic activity of NTG is limited by the array of genetic alterations in the mutants comprising the tester set. To investigate the general mutagenicity of NTG with respect to induction of chromosome breakage as well as point mutants, forward-mutation experi-

Table 22.14.1. Survival Percentage and the Reversion Frequencies Obtained for Each Mutant in the Tester Set After Treatment with NTG

The conidia were treated for 60 min at 25°C with 0.02 nM NTG/liter dissolved in a phosphate buffer at pH 7.0

Mutant No.	Reversion Mechanism	Percent Survival	Reversions per 10^8 Survivors		*P*
			SP	NTG	
2-17-8	?	104	0	5	10^{-2}
2-17-23	?	79	0	0	1.00
5-4-1	+/–	72	0	1	0.30
2-17-7	+/–	80	0	7	10^{-2}
2-17-61	GC → AT	95	0.6	41	10^{-10}
2-17-155	GC → AT	130	0	43	10^{-9}
2-17-18	AT → GC	74	3.5	77	10^{-10}
2-17-126	AT → GC	97	0.7	19	10^{-7}

Notes: GC → AT, AT → GC: revert by a base-pair transition.
+/–: revert by a base-pair insertion or deletion.
?: revert by an unknown mechanism.
P: the probability that the reversion frequencies obtained in the treated and untreated series belong to the same population.
SP: spontaneous reversion frequency in the untreated series.

ments were performed to study inactivation and mutation induction in a genetically marked dikaryon.

In forward-mutation experiments with this system, NTG gave 1.5 *ad-3* mutants per 10^3 survivors at 40% survival. This is a higher forward-mutation frequency than that obtained with any other mutagens in this system. Almost all *ad-3* mutants obtained after the NTG treatment are "point mutations" at the *ad-3A* or *ad-3B* locus. In tests for allelic complementation it was found that 81% of the *ad-3B* mutants have nonpolarized complementation patterns, 1% polarized complementation patterns, and 18% are noncomplementing. From the analysis of the NA-induced mutants, it is found that a mutation from GC to AT more frequently results in mutants with polarized patterns or noncomplementing mutants.[5] The percent complementation after NTG treatment was much higher than among mutants induced by EMS and HA. The last two compounds induce mainly base-pair substitutions from GC → AT. Based on this comparison, we have concluded that NTG reacts predominantly with AT base-pairs, resulting in a base-pair transition from AT → GC. Since A is resistant to diazomethane and T is readily alkylated by this compound, the last reaction is probably most important for the mutagenicity of NTG.

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22.15 MUTAGENICITY OF METHYLHYDROXYLAMINES IN *NEUROSPORA CRASSA*

H. V. Malling

Both hydroxylamine (HA) and its methylated derivatives, methoxylamine (OMHA) and *N*-methylhydroxylamine (NMHA), are mutagenic on the transforming DNA of *Bacillus subtilis*.[1] Hydroxylamine induces base-pair substitutions in phage,

and the type of genetic alteration is preferentially from guanine-cytosine (GC) to adenine-thymine (AT).[2] HA induces reversions in *Neurospora crassa* only in those mutants which revert by a base-pair substitution, and most likely only in those base-pair substitution mutants that revert by a GC → AT transition.[3]

Derivatives of HA and HA itself have versatile biological effects on mammalian systems. Somers and Hsu[4] have found that these compounds induce chromosome damage in mammalian cells, and Borenfreund, Krim, and Bendich[5] have found that NMHA induces the chromosome aberrations. Free arylhydroxylamine derived directly from carcinogenic amines or by deacetylation of carcinogenic *N*-hydroxyamides may be the proximate carcinogens and even ultimately carcinogens in rodents.[6] It is therefore particularly important to study the molecular level. In order to start this study the two simple methylated hydroxylamine derivatives mentioned above were chosen. The system we have used is the reverse mutation system of *Neurospora crassa*. Testing for the induction of reversions in mutants resulting from known genetic alterations is a much simpler method to characterize the genetic effect of mutagens than any known forward-mutation technique in *Neurospora*. The characterization of the mutagenicity is limited by the array of genetic alterations in each mutant comprising a tester set.

The tester set is composed of 14 base-pair substitution mutants which have nonpolarized complementation patterns and 8 noncomplementing mutants which revert by base-pair insertion or deletion.[7] The base-pair substitution mutants were selected among 64 nitrous acid (NA)-induced mutants with nonpolarized complementation pattern in order to cover a wide range of reversion frequencies after HA treatment (Table 22.15.1A). The results in Tables 22.15.1A and 22.15.1B indicate that only the mutants which in earlier tests[7] were characterized as base-pair substitutions can revert with the hydroxylamines. These data suggest that these three compounds only induce base-pair substitutions.

If HA, OMHA, and NMHA possessed the same chemical activity, the reversion frequencies obtained after treatment with these compounds should be positively correlated. It is quite clear in Table 22.15.1A that the reversion frequencies obtained after treatment with HA and OMHA are positively correlated and that a negative correlation exists

Table 22.15.1A. Reversion Frequencies Obtained After Treatment of NA-Induced *ad-3B* Mutants with HA, OMHA, and NMHA. A. Base-Pair Substitution Mutants

Mutant No.	Reversions per 10^8 survivors			P^a		
	HA	OMHA	NMHA	HA	OMHA	NMHA
2-17-22	0	31	408	1.00	10^{-6}	10^{-9}
2-17-129	1	4	164	4.6×10^{-1}	2.3×10^{-2}	10^{-9}
2-17-51	8	7	27	3.5×10^{-3}	5.3×10^{-2}	10^{-9}
2-17-73	11	4	43	2×10^{-4}	2.3×10^{-1}	10^{-9}
2-17-116	8	64	130	7×10^{-3}	10^{-9}	10^{-9}
2-17-68	14	23	312	1×10^{-1}	2×10^{-2}	10^{-9}
2-17-24	16	24	53	6×10^{-4}	10^{-7}	10^{-10}
2-17-155	21	60	63	10^{-6}	10^{-9}	10^{-9}
2-17-35	22	152	74	10^{-9}	10^{-9}	10^{-9}
2-17-130	23	14	60	5×10^{-3}	1.3×10^{-3}	10^{-9}
2-17-148	28	256	73	10^{-9}	10^{-9}	10^{-9}
2-17-17	29	12	206	10^{-10}	10^{-5}	10^{-9}
2-17-9	30	40	8	10^{-7}	10^{-6}	3×10^{-5}
2-17-61	64	38	42	10^{-9}	10^{-8}	10^{-9}

[a]*P* – the probability that the reversion frequencies obtained in the treated and untreated series belong to the same population.

Table 22.15.1B. Reversion Frequencies Obtained After Treatment of NA-Induced *ad-3B* Mutants by HA, OMHA, and NMHA. B. Base-Pair Insertion and Deletion Mutants

Mutant No.	Reversions per 10^8 survivors			P^a		
	HA	OMHA	NMHA	HA	OMHA	NMHA
2-17-13	0	0	0	5.7×10^{-2}	1	1
2-17-36	0	0	1	1.00	1	2.7×10^{-1}
2-17-48	0	0	0	1.00	1	1
2-17-83	4	5	0	2×10^{-1}	1.3×10^{-1}	1
2-17-88	0	0	0	1.00	1	1
2-17-101	1	0	4	2.4×10^{-1}	1	4.4×10^{-2}
2-17-132	1	3	1	1.00	2.3×10^{-2}	4.4×10^{-1}
2-17-162	1	0	0	1.00	1	1

[a]P – the probability that the reversion frequencies obtained in the treated and untreated series belong to the same population.

between the reversion frequencies obtained after treatment with HA and NMHA. We have interpreted this fact as indicating that HA and NMHA might induce mutations by two different mechanisms. HA seems to induce base-pair substitution preferentially from GC → AT in bacteriophage T4,[2] and probably also by the same mechanism in *Neurospora*.[3] The positive correlation between the reversion frequencies obtained after treatment with HA and OMHA indicates that OMHA induces base-pair substitutions like HA from GC → AT. The negative correlation found between the reversion frequencies obtained after treatment with NMHA and HA or OMHA, respectively, and since NMHA only induces base-pair substitution, could be due either to the fact that NMHA preferentially induces mutations from AT → GC or that it induces both types of base-pair transitions.

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22.16 INHALATION CARCINOGENESIS

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Alfred Hellman, Paul Nettesheim
R. F. Newell

The protocol for the inhalation experiment has been fully described in previous reports.[1] Three additional chambers, containing initially 540 C57B1/6 mice each that have been pretreated with 100 r x radiation and infected with PR-8 influenza virus, have been placed in operation. All virus-treated mice were orbitally bled, and only those showing evidence of infection as manifested by H.I. titer at two weeks after infection were selected for chamber use. The current status of the chamber operations is summarized in Table 22.16.1. In all 5400 mice have been used, of which 450 have been sacrificed at chamber startup for initial pathology. Of the 4950 mice that have been placed in the chambers, 216 have died or been sacrificed for examination, and the results of the autopsies of all but the 69 animals sacrificed for a six months examination or electron microscopy are given in Table 22.16.2. There seems to be a

trend toward urogenital disease in the animals exposed to chromic oxide dust and toward a low incidence of gastrointestinal disease in all the virus-treated groups. At six months, sections of lungs of the animals exposed to dust clearly show the chromic oxide in the alveolar macrophages and in desquamated macrophages in the bronchus.

The previously noted hair loss[1] has been observed in all the chambers. Consequently, four additional control groups (VII to X, Table 22.16.1) have been started, with the animals receiving no chamber exposure and being housed in plastic pans. In order to compare these with wire cages, an additional four groups will be set up housed in

Table 22.16.1. Chamber Operations

Chamber No.	Pretreatment	Chemical	Exposure (hr)
I	½ irradiated	0	734
II	½ irradiated	Cr_2O_3, 53 ppm	582
		Cr_2O_3, 27 ppm	363
III	½ irradiated	Ozone, 1 ppm; gasoline, 30 ppm	826
IV	½ irradiated – all virus	Ozone, 1 ppm; gasoline, 30 ppm	522
V	½ irradiated – all virus	Cr_2O_3, 53 ppm	141
		Cr_2O_3, 27 ppm	275
VI	½ irradiated – all virus	0	345
VII[a]	0	45 pans	0
VIII[a]	Irradiated	45 pans	0
IX[a]	Virus	45 pans	0
X[a]	Irradiated – virus	45 pans	0

[a]No exposure in the chambers – kept in plans to compare with chambers I and VI controls.

Table 22.16.2. Gross Autopsy Diagnosis

Chamber Starting Date	I 1-18-66	II 11-15-65	III 12-13-65	IV 2-28-66	V 4-5-66	VI 4-29-66	VII 3-8-66	VIII 4-11-66
Diagnosis								
Hydrocephalus	2		1	1	1		1	2
Thymoma		2	1	1				
Starvation (long teeth)	2	2	2	2	1	1	2	2
Wasting (cause unknown)	1	4		1				
Physical injury (handling, etc.)		7	1					
Dehydration	2							
Urogenital disease	1	13	6	8	2		1	
Gastrointestinal disease		3	1	9	9	8		
Respiratory disease				1	3			
Undetermined	4	16		5		1		
Other		5 (1 Preg.)	2	2	2			3
TOTAL	12	52	14	30	18	10	4	7

chamber cages. The hair loss is independent of any of the treatments to the chamber animals. It begins at 1 to 2 weeks at the back of the head and spreads over the whole back of the animals, reaching a peak at 6 to 8 weeks. Although there is some regrowth of hair the loss persists to the present time. Some 90% of the animals are affected, 50% showing a medium loss, 10 to 30% heavy loss, and the balance light hair loss. There is no demonstrable pathology, bacteriology, or parasitology of the skin. There is at present a low – 4% – incidence of light hair loss in those control animals living in pans. Apparently this is a stress response that may be aggravated by the conditions of chamber living. Experiments to verify this are planned when additional small experimental chambers have been installed.

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22.17 LYMPHATIC TISSUE GERMINAL CENTER LOCALIZATION OF ^{125}I-LABELED HETEROLOGOUS AND ISOLOGOUS MACROGLOBULINS

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Recent reports describing use of both particulate and soluble antigens have demonstrated lymphatic tissue germinal centers as structures involved in localization of antigen. Fluorescent labeled antibody techniques have been used to study the localization of human serum albumin during a secondary response in rabbit spleen. The results demonstrate a persistence of antigen in spleen lymphatic germinal centers. Also, early localization of antigen in primary follicles of rat popliteal lymph nodes locally injected with ^{125}I-labeled *Salmonella flagella* has been described.

The role of lymphatic germinal centers is still a prime question. Our previous autoradiographic studies in young adult mice have shown that an early response within the spleen white pulp after intravenous injection of antigen increased proliferation of cells in germinal centers which were present at the time of injection. This could be detected as early as 2 hr after an intravenous injection of antigen. We have also described histologic changes in the germinal centers during the first 24 hr after antigen which have been shown to be antigen-dose-dependent. These reports describe a dissociation or loss of germinal centers during the latent phase of the hemagglutinin response, followed by recovery and secondary hyperplasia of the centers at later intervals. Thus, systematic changes have been described in germinal centers preexisting at the time of antigen injection.

Since this tissue response seems to be a very fundamental part of the immune reaction, it is important to understand the persistence and localization of antigen during an immune reaction in relation to these known cellular changes. Also, since these germinal centers are responding to previous antigen exposure, studies on antigen localization in these centers could provide information on the ability of centers to respond to more than one antigen. In order to determine the importance of these events, localization studies are required using well-defined antigens for which isologous controls are available. The purpose of the present study was to investigate spleen germinal center localization of antigen during a primary and secondary immune reaction to a well-defined horse macroglobulin using isologous BC_3F_1 male mouse macroglobulin as a control.

Materials and Methods. – Male BC_3F_1, specific pathogen-free (SPF) mice 12 to 14 weeks of age were used.

Preparation of Heterologous (Horse) and Isologous (Mouse) Macroglobulin. – Macroglobulins were prepared by centrifuging 25 to 30 ml of whole serum on a 1700-ml sucrose density gradient on the B-IV zonal centrifuge. Pooled samples from four to six such runs were concentrated and subjected to a second centrifugal separation. The preparations were ultracentrifugally homogeneous in the analytical ultracentrifuge.

Both the heterologous and isologous macroglobulin preparations were labeled with ^{125}I. The final labeled preparations had a specific activity of 11.8 μc/mg. It was estimated that an average of two ^{125}I atoms were tagged to each protein molecule.

Procedure. – Six groups of mice were used in these experiments. All injections were intravenous. Groups 1 and 2, each containing 50 animals, were injected with 0.3 mg ($\sim$3.5 μc) and 3.0 mg ($\sim$35 μc) of ^{125}I horse macroglobulin (HMG). Groups 3 and

4, each containing 50 animals, were injected with 0.3 mg (~3.5 μc) and 3.0 mg (~35 μc) of ^{125}I isologous macroglobulin (IMG). The 50 mice in group 5 were noninjected controls. Twenty mice in group 6 were each injected with 3.0 mg of horse macroglobulin, and six weeks later were injected with 3.0 mg of ^{125}I horse macroglobulin (~35.0 μc).

Hemagglutinin titers were determined from serum using the passive hemagglutination technique. Radioactivity of tissues was measured in an Auto-Gamma counter prior to preparation of the tissue for autoradiography.

Results. *The fate of heterologous and isologous macroglobulins in the mouse.* – The distribution with time of the 3.0- and 0.3-mg doses of ^{125}I-labeled isologous macroglobulin (IMG) and heterologous macroglobulin (HMG) in mouse spleen, liver, and serum was measured. A more rapid clearance of IMG is seen when the percentage label retained in the spleen of mice injected with either dose of IMG is compared with that of mice injected with HMG. At two days after injection, there is fourfold difference, and this was maintained to day 20 after injection.

In the serum, the greater retention of HMG is seen only between days 1 and 4 when the 0.3-mg HMG group is compared with the 0.3-mg IMG group, and between days 2 and 4 when the same 3.0-mg groups are compared.

Hemagglutinin Response to Horse Macroglobulin in Mice. – The primary hemagglutinin response in mice injected with 0.3 mg of HMG and 3.0 mg of HMG and the secondary response in mice injected with 3.0 mg of HMG are shown in Fig. 22.17.1. There was no significant difference ($P > 0.05$) when the primary response of mice injected with 0.3 and 3.0 mg of HMG was compared with the background hemagglutinin response in noninjected mice. A marked secondary hemagglutinin response is measured in serum from mice injected with 3.0 mg of HMG. This response has a latent phase of two days, with the log phase occuring between days 2 and 4. A peak titer of ~11 $\log_2$ units is maintained for 30 days after injection.

Autoradiographic Studies of Fate of ^{125}I Heterologous and Isologous Macroglobulins: 0.3 mg ^{125}I HMG. – In the spleen, the label is primarily in the central arteries and marginal zones of lymphatic nodules within minutes after intravenous injection. By 2 hr the grains were heavily dispersed throughout the spleen. Although the label was mainly associated with the spleen red pulp, random labeling was detected in lymphatic nodules. Observed aggregates of grains in the red pulp at 12 hr indicated phagocytic activity (Fig. 22.17.2*A*). The grains were still mainly in the spleen red pulp at one day; however, the concentration had decreased. A difference in persistence of label in germinal centers in comparison with the associated lymphocyte mass of lymphatic nodules could also be seen at this time. At four days the spleen red pulp was essentially cleared of label; and rather than a persistence of label in germinal centers, there was an enhanced localization of grains in the majority of the centers. This marked localization increased to 15 and 20 days after injection, when approximately 50% of the splenic germinal centers had dense grain concentrations (Fig. 22.17.2*B*). There appeared to be more grains associated with the germinal centers at this time than could originally be seen at 2 hr after antigen.

3.0 mg ^{125}I HMG (Primary Response). – The same labeling pattern was observed in the spleen as that described for the 0.3-mg group. A persistence of label in germinal centers occurred through day 4 with a localization occurring in the centers to day 20 after injection.

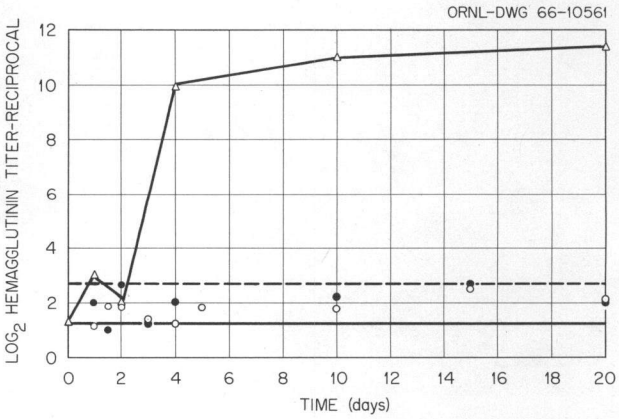

Fig. 22.17.1. Mean Hemagglutinin Response in Mice Intravenously Injected with Primary and Secondary Doses of ^{125}I Horse Macroglobulin (HMG). Five mice per point: ○, 3.0 mg ^{125}I-HMG (primary); ●, 0.3 mg ^{125}I-HMG (primary). Three mice per point: △, 3.0 mg ^{125}I-HMG (secondary). The background titer was determined from 22 noninjected mice. The background is expressed with 2 standard deviations from the mean.

A marked difference in the mesenteric lymph nodes of these mice in comparison with the 0.3-mg group was a retention of label in cortex germinal centers at seven days, and this persistence of label continued to day 20.

0.3 mg and 3.0 mg ^{125}I IMG. – There was a rapid general clearance from all tissue during the first 24 hr after injection. Except for two major differences, the antigen retention pattern in tissue autoradiograms of these mice was comparable with that described in the HMG groups. The significant difference was that at none of the intervals, in neither the spleen nor the mesenteric lymph node, could any persistence or localization of grains be seen in lymphatic tissue germinal centers. The second difference was that during the first 24 hr, when clumps of grains could clearly be seen in macrophages of spleen red pulp in the HMG groups, they were infrequently seen in the spleen of animals injected with ^{125}I IMG.

Discussion. – A significant finding in these studies is that after primary injection of 0.3- and 3.0-mg doses of horse macroglobulin, where there is no detectable serum antibody and no marked immune clearance of antigen in tissue or serum, there is a persistence of antigen in splenic ger-

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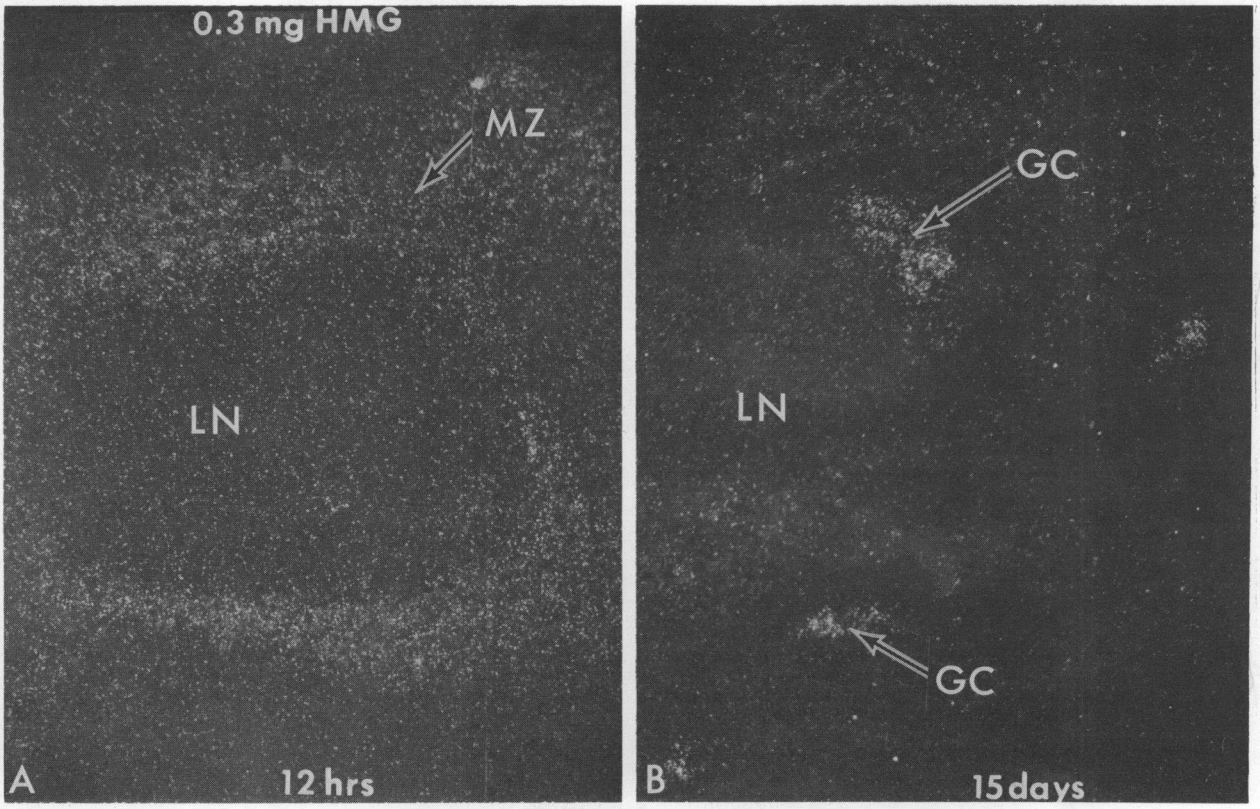

LN LYMPHATIC NODULE
MZ MARGINAL ZONE
GC GERMINAL CENTER

Fig. 22.17.2. Localization of Grains in Spleen Autoradiograms of Mice Injected with 0.3 mg ^{125}I Horse Macroglobulin (HMG). The photographs were taken using a dark-field condenser in order to more clearly demonstrate grain pattern.

minal centers to day 4, followed by an enhanced localization of antigen to day 20. Without appropriate isologous protein control, we could have justifiably concluded that these mice were "unresponsive" to a primary injection of horse macroglobulin. Isologous controls were included in this study. Animals that were injected with immunogenically inert protein of the same molecular weight and possibly tertiary structure as the test antigen also failed to respond in terms of the two conventional immunological parameters. However, the characteristic antigen localization pattern in the germinal centers was not seen in these animals. These histologic studies suggest that in mice injected with the test antigen, immunologic reactivity occurred at the cellular level in absence of detectable reactivity at the serologic level.

One possible explanation for this is redistribution of antigen from nonsplenic tissue which is trapped by the dendritic macrophages of the germinal centers. The present studies, as well as others, show that the bulk of the injected antigen is retained in the liver. Recent studies have shown that different tissues become involved to a varying degree with the handling of antigen and that some antigen localized initially in liver tissue emigrates and is redistributed as a result of subsequent injection of specific antigen. The enhanced localization of antigen at later intervals described in the present study could be a normal reflection of this process.

References

[1]Radiation Immunology section.
[2]General Physiology section.

22.18 PROLONGED ANTIBODY SYNTHESIS OF SPLEEN CELLS CULTURED IN DIFFUSION CHAMBERS

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Introduction. – We recently reported on the improvements of the Millipore diffusion chamber technique for quantitative cytokinetic analysis of antibody response. Long-term studies of sustained antibody production were not possible until now, since culture conditions began to deteriorate approximately four weeks after culture.

Methods and Results. – In order to establish conditions which would support antibody formation for extended time periods, the following experiments were carried out: Chambers containing 12.0×10^6 primed spleen cells and an optimum amount of the test antigen (sheep RBC) were cultured in the peritoneal cavity of 600 r irradiated isologous recipients. On days 6 and 10 they were removed from the first hosts and implanted in second recipients. Serum samples were taken from the second recipients at intervals after transfer of the chambers and titrated. The results are summarized in Fig. 22.18.1. It can be seen that within 24 hr after transfer of the chambers into the second recipient, the serum antibody titer reached the level of that observed in the primary recipient. After a brief overshoot, the titers returned to a "normal" level extending up to 16 and 20 days after antigenic stimulation, at which time the experiments were terminated. The rapid rise in the

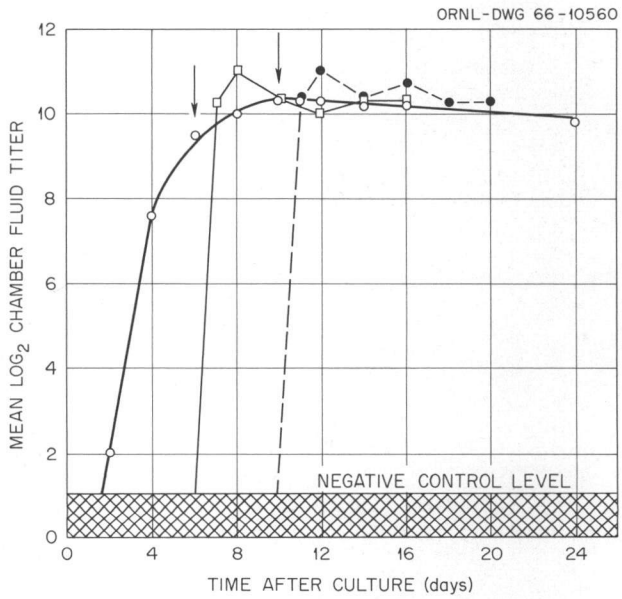

Fig. 22.18.1. Transfer of Chambers from the First to a Second Host Six and Ten Days After Initiation of Secondary Antibody Response. Each chamber contained 12.0×10^6 primed spleen cells and 1.2×10^6 sheep RBC. ○, typical secondary response profile; secondary response profile after transfer of chambers into a second host six days (□) and ten days (●) after culture. The arrows indicate time of transfer. Cross-hatched area indicates level of naturally occurring sheep RBC hemagglutinins in 600-r irradiated controls; three to five samples per point.

antibody titer up to the normal level shortly after transfer, in the face of an approximate tenfold dilution factor (body fluid volume of the second recipient), and the sustained antibody level for the following nine days after transfer clearly indicate that the stationary-phase cultures were actively synthesizing antibody after their transfer into secondary hosts.

In a subsequent experiment, chambers containing spleen cells and antigen were transferred to new irradiated recipients every other week. The chamber surfaces were wiped free of adhering peritoneal cells between transfers. Serum antibody titers were determined.

As summarized in Table 22.18.1 antibody is detectable in the serum of the chamber recipients for more than 14 weeks. The persistent serum antibody level cannot be accounted for simply by release from antibody contained in the chambers, since each chamber transfer would cause an approximate tenfold decrease in chamber antibody concentration. With the six consecutive transfers, this would result in a dilution factor of about 10^6. Therefore, it must be concluded that antibody is continuously synthesised, though in decreasing amounts, over at least a 3.5 months period.

Whether long-living plasma cells are responsible for the continued antibody synthesis or a continuous proliferation with new formation of plasma cells has yet to be determined.

Table 22.18.1. Continued Antibody Synthesis by Serially Transferred Diffusion Chamber Cultures

Weeks After Culture	Serum Titer
2	9.4
4	8.7
6	8.0
8	7.8
10	6.7
12	4.6
14	5.0

References

[1]Radiation Immunology section.

22.19 CHROMOSOMAL ABERRATIONS IN CELLS FOLLOWING INFECTION WITH VIRUS

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Chromosome aberrations have been observed in cells in vitro subsequent to infection with herpes simplex virus,[2,3] human adenovirus,[4] and in blood cells of measles patients.[5] With the possible exception of this report involving measles virus, information pertaining to the effects of RNA virus infection on chromosome integrity has not been published. Over the past several years we have been studying the influence of polio virus infection on chromosome breaks in HeLa cells.

Table 22.19.1. Chromosomal Aberrations in HeLa Cells Following Infection with Polio Virus

Hours After Addition of Polio Virus	Total Number of Metaphase Plates Counted	Number of Plates with Breaks	Percentage of Plates Counted with Breaks
Control	130	9	6.7
7	115	7	6.1
8	118	9	7.6
9	110	8	7.3
10	128	15	11.7
11	123	17	13.8
12	115	25	21.7
13	120	28	23.3
14	lyse		
15	lyse		

Chromosome preparations were made utilizing the squash technique. Cells were fixed for study at time intervals ranging from 1 to 14 hr after viral infection. In control cultures 6.7% of the cells scored had at least one chromosome break. These cells averaged 65 chromosomes per metaphase plate. Results of virus infection for different time intervals are shown in Table 22.19.1.

The mechanism of chromosomal breakage following viral infection has not been elucidated. In order to find more definitive answers, kidney cells from two species of monkeys susceptible to polio infection are currently being investigated. Initial data indicate that polio virus also produces an effect on the chromosomes of these diploid cells. Results from this endeavor are still preliminary and await further cytological and biochemical evaluation.

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22.20 AMINOACYL-tRNA SYNTHETASE SYSTEMS OF VIRAL INFECTED CELLS

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Our studies on viral-induced alterations on host cell aminoacyl-tRNA synthetase are continuing. We have determined both quantitative and qualitative parameters of viral-induced synthetase alterations. Eighteen aminoacyl-tRNA synthetases are being investigated, and preliminary results indicate quantitative viral-induced synthetase differences.[1] Temperature sensitivity of normal HeLa cells and viral-infected cells is currently under study.

Countercurrent separations of tRNA of HeLa and viral-infected HeLa cells are now being carried out, as are studies in the isolation of and characterization of specific aminoacyl-tRNA synthetases. This has now become possible due to the development of procedures for the growth of mammalian cells in vitro in large quantities.[2]

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22.21 PHOTODYNAMIC ACTION OF BENZO(*A*)PYRENE AND 355-mμ ILLUMINATION ON *ESCHERICHIA COLI*

A. P. Harrison, Jr. Vivian E. Raabe

Death of *E. coli* results when a buffer suspension is exposed simultaneously to colloidal benzo(*a*)-pyrene and 355-mμ illumination. Neither hydrocarbon nor illumination alone causes death, and oxygen must be present. The survival curve has a shoulder; then death proceeds exponentially, and DNA is degraded. Death rate is independent of temperature between 6 and 32°C. The duration of the shoulder, however, decreases somewhat with rise in temperature.

The amount of ^{3}H-benzo(*a*)pyrene tightly bound to the cells was determined after three ethanol:ether extractions. Appreciable binding occurs in the presence of 355-mμ illumination with air, but relatively little binding occurs under nitrogen; almost no tight binding occurs in the dark either with nitrogen or with air. Hydrocarbon binding ceases the instant the suspension is transferred to the dark. The hydrocarbon, once bound, is not released from the cells during growth in the dark. At a low 355-mμ dose rate, death ceases the instant the suspension is transferred to the dark. At 80 ergs mm^{-2} sec^{-1} and above, death may continue in the dark. Alternations between darkness and 355-mμ illumination cause a response which suggests the presence of dark-labile photochemical products. There is some evidence for cell recovery.

Cell lysis does not occur: turbidity does not change, and cells labeled with ^{3}H-thymidine do not lose label to the menstruum. However, protein (or protein derivatives) elutes from the cells.

Scission of bacterial DNA occurs, but scission itself probably is not the cause of death: Inactivation of intracellular phage takes place not at the low rate characteristic of free-phage inactivation, but at the same rapid rate characteristic of host cell death. Thus, photodynamic damage to the host prevents phage maturation. If damage were solely to host DNA, phage maturation should not be disturbed.

Mutation (from auxotrophy to prototrophy and from phage sensitivity to phage resistance) by *E. coli* has been sought, but never detected, with benzo-(*a*)pyrene, either in the dark or under 355-mμ illumination.

22.22 METABOLIC STUDIES ON 1-(4-DIMETHYLAMINOBENZYLIDENE)-INDENE

C. T. Bahner D. G. Doherty

Study of the fate of 1-(4-dimethylaminobenzylidene)-indene in vivo is being continued, using compound labeled with ^{14}C in a methyl group. In Sprague-Dawley rats fecal excretion of radioactivity following oral administration fell off more rapidly than following ip administration, and urinary excretion of activity was less than half as great as after ip administration; also, the specific activity of liver tissue was only one-fourth as high 72 hr after oral administration as after ip administration. These facts suggest that the greater antitumor effect of a single dose of the compound when administered ip rather than orally may be due to more prolonged contact with the tumor.

Fischer rats which received the compound ip excreted less than half as much radioactivity in the urine during the next 72 hr as Sprague-Dawley rats did, and the same difference was noted in fecal excretion, but the specific activity of the Fischer rat liver tissue was twice as great. These facts may account for the fact that the compound is somewhat more toxic to Fischer rats than to Lewis strain Wistar rats. Small Dunning IRC-741 tumors in the Fischer rats had specific activities less than liver and spleen, but several times as great as muscle. The moderate uptake of radioactivity may be related to the fact that this tumor is relatively unresponsive to the compound, in contrast to the Walker 256 tumor and Lymphoma 8, in Lewis rats. The concentration of the drug in the latter two types of tumor should be investigated.

Chromatographic patterns of urinary metabolites from the three sets of rats mentioned above are similar, although some differences may exist. In each case a large part of the ^{14}C in the first-day urine is found in the urea spot, indicating that some demethylation has taken place and suggesting that part of the ^{14}C is converted to CO_2, as is known to be the case with certain other dimethylamino compounds.

The first autoradiograms of tissues from animals which have received the radioactive compound have become available. Since the free base is soluble in alcohol and in xylene, it tends to be removed during preparation of slides by conventional methods, but firmly bound radioactivity remains. An unexpected observation was the relatively high concentration of radioactivity in the outer nuclear layer of the retina in comparison with other parts of the eye. Less surprising was the concentration of activity in lymph nodes and in myelocytes of the bone marrow, a finding probably related to the effectiveness of the drug against the Lymphoma 8. With increasing experience in dealing with this compound, autoradiograms should provide further valuable information.

AEC-NIH MOLECULAR ANATOMY PROGRAM

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22.23 STUDIES ON NATIVE GLYCOGEN ISOLATED BY COMBINED RATE-ZONAL AND ISOPYCNIC ZONAL CENTRIFUGATION

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Introduction. – Rat liver glycogen exists in a continuous spectrum of particle sizes ranging up to 150 to 200 mμ. A gradual decrease in concentration is noted with increasing particle sizes.[3] The particles can be isolated in samples each having a uniform particle size by rate-zonal centrifugation in density gradients. Several experimental approaches have now been used to determine the metabolic homogeneity of glycogen as related to particle size. These include measuring the particle size spectrum in starved and refed animals as well as the distribution of phosphorylase and UDPG-glycogen transferase activity in the glycogen particles of various sizes isolated by rate-zonal centrifugation. Rate-zonal centrifugation of homogenates also results in the separation of large amounts of membrane-associated glycogen. Further studies have been carried out to characterize this association of glycogen with membranes.

Results and Discussion. – In normal animals a range of particle sizes was observed, with a

gradual increase in concentration noted with increasing particle size. In starved animals there was a rapid decrease in total glycogen. All particle sizes were found to be utilized during the starvation period. By 24 hr, 85% of the total glycogen was depleted and only small particles remained. In refed animals, total glycogen was rapidly restored, exceeding that of controls by 24 hr. Restoration of both the larger and smaller particles was noted during the early stages of refeeding. The initial increase in concentration of smaller particles was rapid, exceeding control values after 4 hr. The small-particle glycogen concentration decreased thereafter as large particles reappeared but remained above normal after 24 hr of refeeding. The concentration of large particles did not return to normal levels by 24 hr. To measure glycogen synthesis, ^{14}C-labeled glucose was injected intraperitoneally into normal and starved rats. Incorporation was measured in glycogen isolated by the isopycnic banding of rate-separated fractions. These studies demonstrated a uniform rate of glucose incorporation into all particle sizes in normal animals. In starved animals, radioactivity was higher in the larger particle sizes during the initial stages of refeeding. These observations suggest rapid formation and reduced turnover of large glycogen particles when starved animals are refed.

Phosphorylase activity in homogenates is normally associated with the glycogen particles. Starvation leads to the solubilization of this enzyme, and reattachment occurs during refeeding. The enzyme appears to be of slightly higher specific activity (micromoles/min per milligram of glycogen) in the smaller glycogen particles than in the larger aggregates. Transferase activity is also associated with the glycogen particles. However, its specific activity increases slightly with increasing particle size. It appears, therefore, that there is a different distribution for the glycolytic and synthesizing enzymes responsible for glycogen metabolism among the different particle sizes.

The association of particulate glycogen with cellular membranes appears to be an artifact related to the centrifugal methods employed for separating the glycogen in density gradients. This was demonstrated as follows: low-speed membrane preparations, nearly free of carbohydrate-containing material, were prepared from liver homogenates. Native glycogen, free of membranes, was prepared

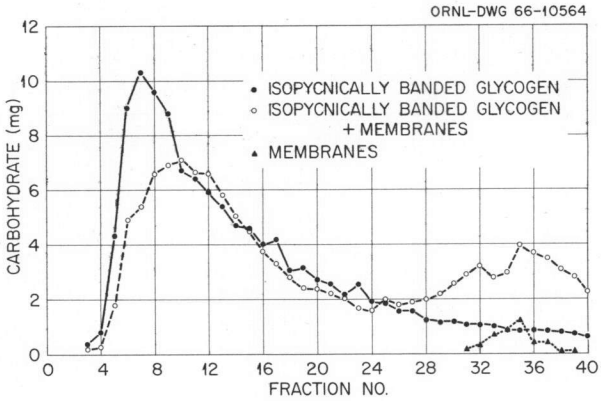

Fig. 22.23.1. Comparison of Distribution of Isopycnally Banded Glycogen in a Rate Zonal Experiment With and Without Added Cytomembranes.

by isopycnically banding liver homogenates in CsCl. These two fractions were combined and placed over a CsCl gradient for a rate-zonal separation of the glycogen particles. The results of centrifugation clearly demonstrate the membrane-associated glycogen as a physical association developed by simply combining the pure glycogen with the membrane fraction (Fig. 22.23.1). These results appear to eliminate much of the physiological significance formerly ascribed to the membrane-associated glycogen fraction of liver.

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22.24 CELLULAR SITES OF SYNTHESIS IN *EUGLENA GRACILIS*

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Introduction. – A study is in progress to determine the early sites of synthesis and protein in log-phase *Euglena*. The major efforts to date have been to define the subcellular particulates

present after homogenization of the cells in various concentrations of divalent ion and their sedimentation behavior in the analytical ultracentrifuge and in sucrose density gradients. In addition, the log-phase cells have been grown in the presence of ^{32}P and/or ^{14}C bicarbonate for various lengths of time, and the distribution of label has been followed as a function of the size of the incorporating particle.

Methods and Results. – Log-phase *Euglena* were grown in modified Cramer-Meyers (C-M) medium[2] to a cell count of 10^5 per milliliter. The cells were concentrated by centrifugation, washed twice with C-M without supplements, diluted to a concentration of 2 ml of packed cells per 25 ml of solution with phosphate-free C-M medium. Pulse-labeling experiments used 2 mc of ^{32}P and/or 25 μc of ^{14}C bicarbonate per 25 ml of suspension. Incorporation was slowed or stopped at designated times by making the suspension 10^{-2} *M* with sodium cyanide and chilling the incubation tubes. The labeled cells were washed twice with cold 1/15 *M* phosphate buffer and finally suspended in 0.01 *M* Tris–0.086 *M* KCl buffers containing $MgCl_2$ at concentrations from 0 to 10^{-2} *M*. The pulse-labeled cells were disrupted in an ice-cold French cell press at 10,000 psig and centrifuged for 5 min at 1000 × *g*, and the supernatant was used as starting sample for the sucrose-density-gradient separations in the B-XV zonal rotor. Unlabeled fractions were carried through the incubation times, fractionated in the French cell press, and concentrated by centrifugation; the resultant pellet was used for analysis in the model E centrifuge.

The labeled samples were centrifuged through 10 to 30% sucrose gradient containing the same buffer and magnesium concentration of the starting sample. The time-gravity factor of each of the separations was 2×10^{11} G_c (about 18 hr at 10,000 rpm). The gradient was collected in 40-ml fractions. Each fraction was diluted with buffer and centrifuged in the No. 30 rotor for 12 hr. Pellets were suspended in buffer, and 100 λ of the suspension adsorbed to filter paper disks and precipitated with cold 10% TCA. The TCA-washed ethanol-ether-extracted filter paper disks were counted in the Beckman scintillation spectrometer in a toluene-PPO-dimethyl POPOP system.

Figure 22.24.1 shows the results of schlieren analysis of classes of subcellular particles of *Euglena* which fall into the size range of ribosomes. Part *a* shows a schlieren photograph of particles obtained using Tris buffer without added magnesium; part *b* shows the same material at

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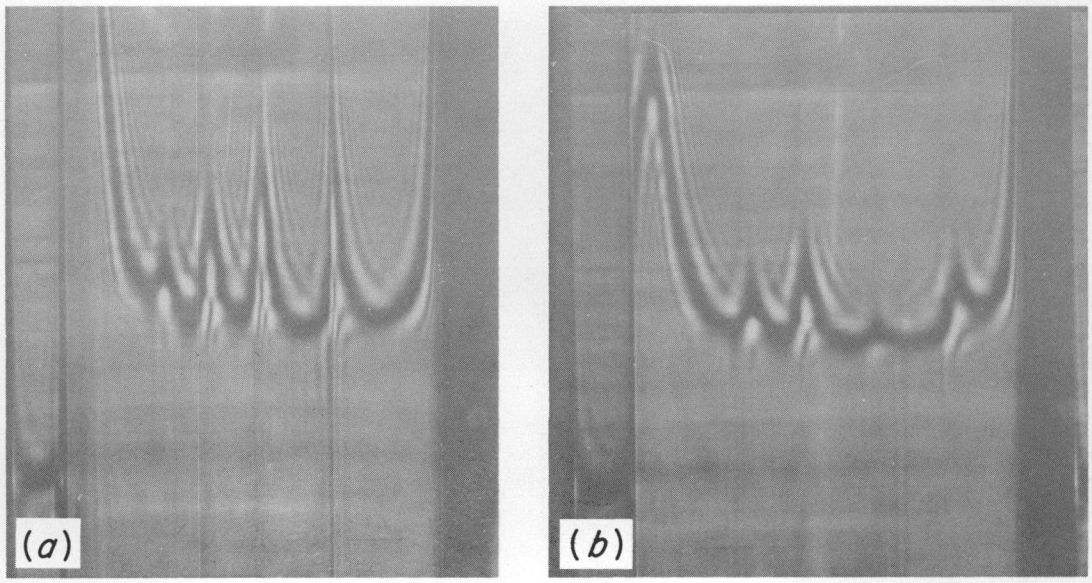

Fig. 22.24.1. A Schlieren Analysis of a Concentrate of Particles of Ribosomal Dimensions from *Euglena*. Part *a* shows the starting concentration. Part *b* shows the results of decreasing the concentration by ½. Observed values of the peaks in *b* are 81, 62, 45, and 30.

one-half the concentration of part *a*. The observed *S* values for the peaks shown in part *b* are 81, 62, 45, and 30. Figure 22.24.2 shows the distribution of TCA-precipitable ^{32}P and ^{14}C bicarbonate on subcellular particulates after a 1-hr incubation.

Discussion. – The disruption of *Euglena* in the French cell press releases a series of subcellular particulates which can be isolated in quantity in large-volume sucrose density gradients in zonal centrifuges. Preliminary schlieren analysis indicates that several classes of these particulates fall into the range for ribosomes. Density gradient separations show a much larger size of particles to also be present. A preliminary electron-microscopic examination suggests that this faster moving zone of material is composed primarily of membrane-ribosome complexes. The distribution of pulse label on all cellular particulates suggests that this cellular fraction is one of the earliest sites of synthesis in the cell. Additional experiments in progress are following the incorporation into *Euglena* in 2, 5, 10, 15, 30 min and 1 hr as a function of magnesium concentration (0, 10^{-4}, 10^{-2} *M*). It is anticipated that this study will indicate the earliest site(s) of synthesis of nucleic acid and protein, show the rate of synthesis on each species of subcellular particle, and indicate the rate of release and/or transfer of pulse-labeled components from one cell fraction to another.

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[2]M. Cramer and J. Meyers, *Arch. Mikrobiol.* **17**, 384 (1952).

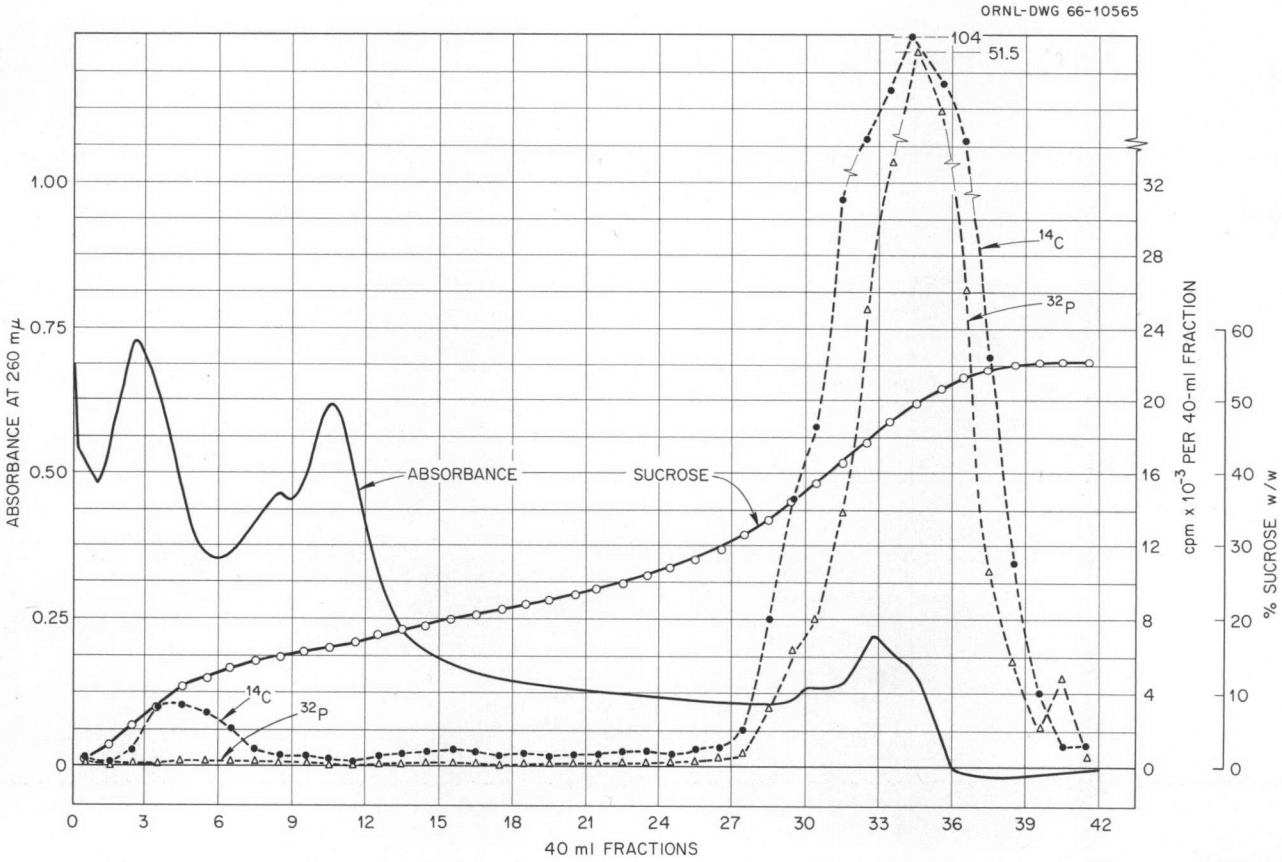

Fig. 22.24.2. Distribution of TCA-Precipitable ^{32}P and ^{14}C-Bicarbonate Particulates from *Euglena* Following a 1-hr Incorporation. The fractionation of the *Euglena* homogenate was carried out on 10 to 30% sucrose gradients for 16 hr at 18,000 rpm in the B-XV zonal rotor.

22.25 ULTRACENTRIFUGATION STUDY OF TUMOR ANTIGENS

R. M. Jamison N. G. Anderson

Hamsters carrying tumors induced by papovaviruses or adenoviruses develop antibodies capable of detecting new antigens (T) in the transformed cells.[1] These new antigens can be demonstrated by complement-fixing tests,[1] by immunofluorescence,[2] and by in vivo immunity tests.[3] Recently, they have been demonstrated in infected nontumor cells early in the replication cycle of the tumor viruses.[4,5] This preliminary report represents the beginning of efforts to isolate and characterize the T antigen(s) of papovavirus SV 40 and human adenovirus 12.

Materials. – Three tumor lines are being investigated: (1) transplants from a hamster tumor caused by papovavirus SV 40, (2) transplants from a hamster tumor caused by human adenovirus 12, and (3) transplants from a nonviral spontaneous hamster tumor. Liver and muscle excised from normal hamsters serve as control tissues.

Methods. – The tissues (25 g/run) were homogenized in a blender at high speed. The gross debris was removed from the homogenate by centrifugation at 2000 rpm in the International PR-2 centrifuge. The supernatant fluid was then centrifuged for 2 hr at 24,000 rpm in the Spinco model L2 centrifuge, and the resultant pellet was resuspended in 40 ml of 0.15 *M* sodium citrate. This suspension was then loaded into the B-XV rotor in the zonal ultracentrifuge and processed according to the standard S-ρ technique.[6] Density beads were utilized as markers for purposes of comparison.

Results. – Figure 22.25.1, a composite drawn from photographs taken after banding, demonstrates results of typical experiments. It can be seen that each of the tumor tissues (*a*,*b*,*c*) contains more material with a density of <1.09 g/cm^3

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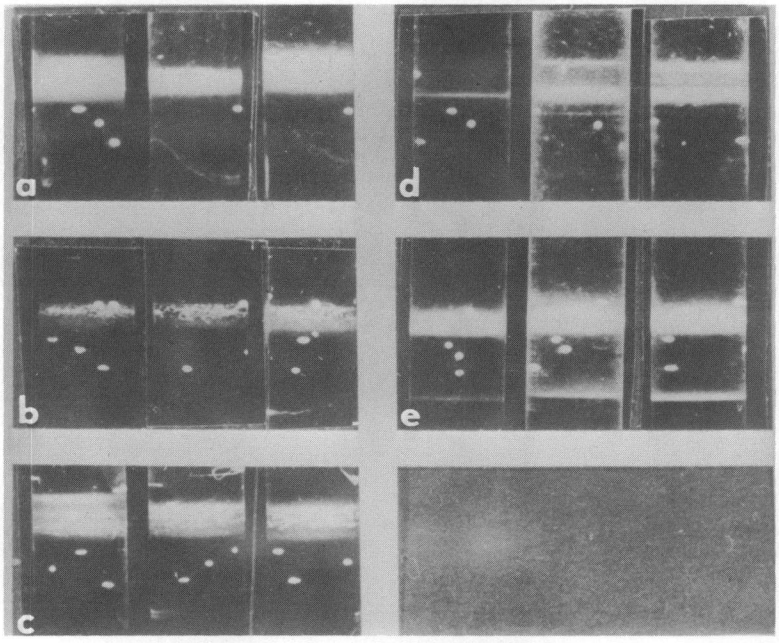

Fig. 22.25.1. Composite Drawn from Photographs Taken After Various Tissue Homogenates Had Been Processed According to the Standard S-ρ Technique. In all cases, the three tubes are comparable. (i.e., tube 1 (*a*) is comparable to tube 1 in each of the other experiments). Figure *a* is a photograph of tubes 6, 7, 8 after centrifugation of a hamster tumor originally caused by papovavirus SV 40. The materials contained in these tubes represent all material with a sedimentation coefficient of $\leqq 280$. (*b*) represents the results of centrifugation of a tumor caused by human adenovirus 12, (*c*) represents the results obtained by centrifugation of a spontaneous nonviral hamster tumor, (*d*) demonstrates results obtained by centrifugation of homogenates of normal hamster muscle, and (*e*) represents the results obtained using homogenates of normal hamster liver.

than the control tissues (*d,e*). It is of interest that the distribution of the densities of the materials in each of the homogenates is different although consistent for any given tissue. Titrations of tubes collected from the *S-ρ* centrifugations indicate that the papovavirus T antigen(s) has a sedimentation coefficient of <30.

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AEC-NASA SPACE BIOLOGY

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Space Biology – Biosatellite (22.26–22.29)

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22.26 SURVIVAL OF *NEUROSPORA* CONIDIA IN SIMULATED THREE-DAY BIOSATELLITE HARDWARE

F. J. de Serres B. B. Webber

Introduction. – During the course of the *Neurospora* three-day Biosatellite experiment, conidia will be sealed in the flight hardware for a five- to seven-day period covering prelaunch preparations, a three-day flight, and postlaunch recovery operations. Since the *Neurospora* package is sealed and the air supply is limited, we have tried to determine whether the conidia could become anaerobic during this period and present a problem with regard either to the response to radiation and/or to the survival of the heterokaryotic fraction. To evaluate this situation, samples of conidia from a genetically marked dikaryon were collected on Millipore filters and inserted into prototype modules. These modules were each sealed in a simulated housing (made out of Pyrex and fitted with a gas sampling port), and seven of these packages were incubated at room temperature. The air was sampled from one package each day for a period of seven days and analyzed by a mass spectrometer. The packages were then opened, and conidia from one or two of the Millipore filters were suspended in water and plated in minimal and complete medium to determine the survival of the heterokaryotic conidia and the total conidial population respectively. Packages were then resealed so that conidial samples could be plated to determine survival levels again on days 10 and 11.

Results. – Analysis of the air content of the simulated flight packages over a seven-day period showed that the oxygen content dropped to 16 to 17% and the CO_2 content increased to 4 to 5% during the first 24-hr period with no subsequent change up to seven days' incubation at room temperature (76 to 78°F). Data from the conidial platings (Fig. 22.26.1) showed that the survival of the heterokaryotic conidia decreased slowly over this same period and showed furthermore then even with 11 days' incubation about 87% were viable. However, by 11 days the survival of the total population had decreased to the point that the heterokaryotic conidia were essentially the only conidia viable.

Discussion. – The results of this experiment indicate that (1) there should be no problem with the *Neurospora* conidia with regard to anaerobiosis during the course of the three-day Biosatellite experiment and (2) that the viability of the heterokaryotic conidia is high, well beyond the anticipated storage time. (The decrease in survival of the total conidial population to the point where only

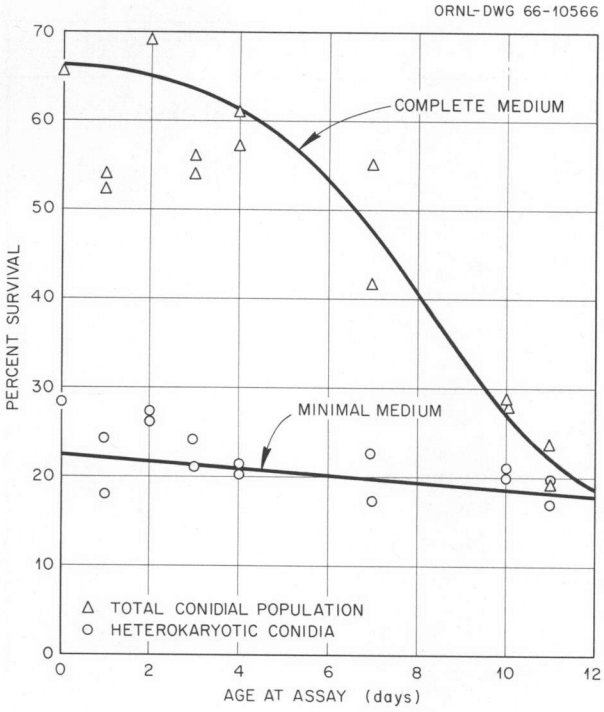

Fig. 22.26.1. Survival of Conidia of a Genetically Marked Two-Component Heterokaryon of *Neurospora crassa* in Simulated Three-Day Biosatellite Hardware at Room Temperature.

the heterokaryotic conidia are viable is not unexpected. Since each component of the dikaryon used for this experiment has a different vitamin requirement, conidia homokaryotic for either genotype will die rapidly under conditions where this requirement is not satisfied.)

22.27 BIOCOMPATIBILITY TESTS OF FLIGHT HARDWARE FOR THE *NEUROSPORA* THREE-DAY BIOSATELLITE EXPERIMENT

F. J. de Serres B. B. Webber

Assurance that the flight-type hardware will support the *Neurospora* three-day Biosatellite experiment is being sought in a series of tests where various associations with flight are simulated either at Ames Research Center (Moffett Field, California) or at General Electric (Philadelphia). Samples of *Neurospora* conidia from the heterokaryon to be used for this experiment were also included in the tests so that biocompatibility data could be obtained simultaneously. At General Electric a series of four tests, (1) vibration, (2) thermal and vacuum, (3) acoustic noise, and (4) reentry shock, were planned for each of the three sets of hardware to be used for the ground control (201) or flight (301 and 302) experiments.

Conidia were packaged untreated or after exposure to 5000 r x rays (~500 to 1000 r/min). Assays were made after each test to determine the viability and survival of the control and irradiated conidia on minimal medium (to estimate the heterokaryotic fraction) and on supplemented medium (to estimate the two homokaryotic fractions as well as the total population). In addition, in some cases forward-mutation experiments were performed to determine the frequency of mutation in the *ad-3* region.

In the Ames test (June 1966) an experiment was performed with an ^{85}Sr source, and assays for survival and forward mutation were made on conidia (1) irradiated, (2) irradiated + simulated flight profile, (3) simulated flight profile, or (4) no treatment.

Results. – The primary assay in each test is to determine the viability of the heterokaryotic conidia. The data for each test performed to date are plotted in Fig. 22.27.1. Although the data are not so numerous as might be desirable, the differences observed have been interpreted as follows:

1. The viability of the heterokaryotic conidia is lower in some of these tests than is expected from the storage test (see Sect. 22.26).
2. All data from the thermal-vacuum tests (T/V) fall on the same curve (*A*), where the viability of the heterokaryon conidia is high.
3. Any data from the vibration tests (Vib) fall on a second curve (*B*), where the viability of the heterokaryon conidia is somewhat lower.
4. The data from the Ames test fall on a third curve (*C*), where the viability of the heterokaryon conidia is the lowest.

Discussion. – Since the Vib tests and the Ames test were done on new hardware and the T/V tests on hardware that had been used previously for other tests, the data suggest a package bioincompatibility problem. Since plastics are known to

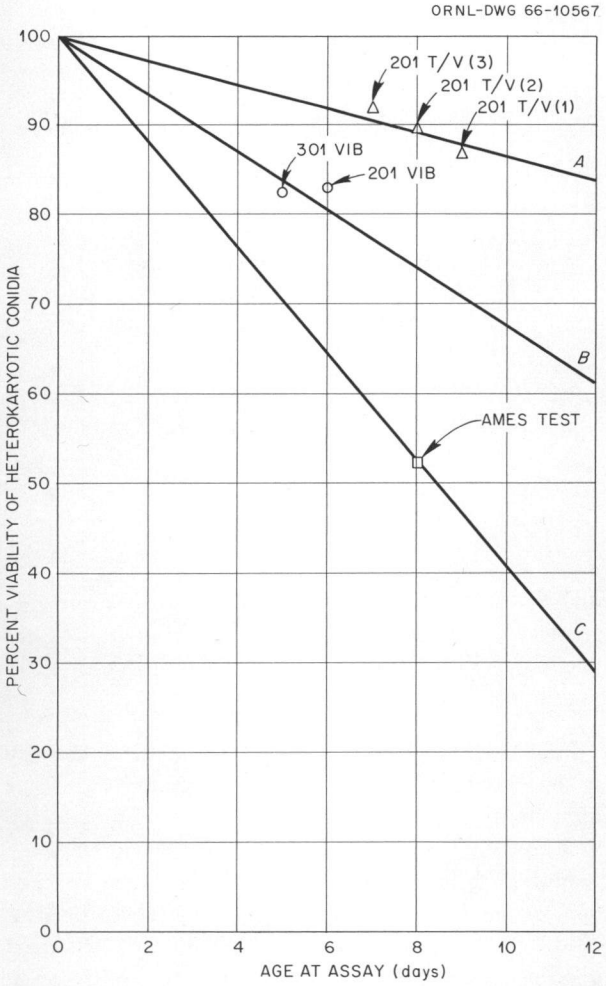

Fig. 22.27.1. Survival of the Heterokaryotic Conidia from a Genetically Marked Heterokaryon in Various Biocompatibility Tests in Ground Control or Flight Hardware for the NASA Three-Day Biosatellite Experiment.

release toxic products during sterilization by autoclaving, it is possible that the lower viability in the Vib tests and the Ames test were due to 24- and 65-hr exposures to such products respectively. Storage experiments on new hardware are planned to verify these conclusions and to determine the most effective procedure to eliminate this problem before new hardware is used for more critical experimentation.

22.28 A SIMULATED SPACE-FLIGHT TEST FOR *HABROBRACON*

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Introduction. – The purpose of this experiment was to test the radiation subassembly and flight packages under two separate conditions: the simulated flight profile with different exposures of radiation including nominal zero dose and the different exposures of radiation including nominal zero dose but without the simulated flight profile. Since this was to be the ground control experiment before the proposed launch of the Biosatellite, pretested females and newly emerged males were employed.

Experimental Conditions. – The test was begun on June 17 and ended on June 20. Before and during the test, careful dosimetric monitoring of the ^{85}Sr radiation source was done with the flight packages in place in the radiation subassembly.

Results. – The summary data for irradiated sperm are listed in Table 22.28.1. The measured exposures are from Toshiba glass rod monitoring during the experiment. As in the April 1965[2] and the August 1966[3] tests, no synergistic effect was observed for the flight profile and radiation for induced dominant lethality in sperm.

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[1]Faculty of Medicine, Osaka University, Osaka, Japan.

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Table 22.28.1. Dose vs Survival of Embryos After Exposure of Sperm to an ^{85}Sr Radiation Source

Nominal Dose (rads)	Radiation Alone		Flight Profile and Radiation	
	Measured Exposure (r)	Embryo Survival	Measured Exposure (r)	Embryo Survival
0	3.06	0.91	1.72	0.92
500	578	0.76	545	0.82
1000	1080	0.78	1030	0.72
2000	1880	0.49	1850	0.59
4000	3870	0.17	3760	0.23

22.29 BIOCOMPATIBILITY TESTS OF *HABROBRACON* UNDER SPACE-FLIGHT CONDITIONS IN THE FLIGHT VEHICLE

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Introduction. – Three flight vehicles are being prepared by General Electric Corporation for NASA: the qualification, the prime flight, and the backup vehicles. Separate hardware and experiment packages have been prepared for each. During the tests for simulated launch vibrations, acoustical noise, and vacuum and thermal conditions, NASA officials have asked the experimenters to place their organisms in the flight packages to ensure that the organisms can withstand the expected flight conditions.

Results. – At this time three biocompatibility tests have been successfully completed: the qualification vehicle vibration, qualification vehicle thermal and vacuum, and the backup vehicle vibration. In all these tests, more than 90% of the *Habrobracon* survived. At present it is adjudged that no untoward conditions exist that should cause cancellation or severe modification of the *Habrobracon* portion of the Biosatellite experiment.

Reports previously issued in this series are as follows:

ORNL-13	Period Ending February 29, 1948
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ORNL-2060	Period Ending February 15, 1956
ORNL-2155	Period Ending August 15, 1956
ORNL-2267	Period Ending February 15, 1957
ORNL-2390	Period Ending August 15, 1957
ORNL-2481	Period Ending February 15, 1958
ORNL-2593	Period Ending August 15, 1958
ORNL-2702	Period Ending February 15, 1959
ORNL-2813	Period Ending August 15, 1959
ORNL-2913	Period Ending February 15, 1960
ORNL-2997	Period Ending August 15, 1960
ORNL-3095	Period Ending February 15, 1961
ORNL-3201	Period Ending August 15, 1961
ORNL-3267	Period Ending February 15, 1962
ORNL-3352	Period Ending August 15, 1962
ORNL-3427	Period Ending February 15, 1963
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