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UNITED STATES
ATOMIC ENERGY COMMISSION

OAK RIDGE OPERATIONS
P.O. BOX E
OAK RIDGE, TENNESSEE 37830

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REPOSITORY Oak Ridge Operations
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Documents 1944-1994
BOX No. H-93-17 Bldg. 2714-H
Cont 4002
FOLDER Texas CA 12-1-69

Ralph Elson, Director, Contract Division
REQUEST FOR CONTRACT ACTION

It is requested that you take the necessary steps to process the following described contract action (CA):

1. Nature of Action Requested:

- Selection of New Contractor and/or Negotiation of Contract
Number: AT-(40-1)-4002
Contractor: Texas Technological College
- Modification of Contract
Number:
Contractor:

2. Nature of Services To Be Covered by Contract: Research

Title: "The Effects of X-Irradiation on the Human Testis: An Electron Microscopic Cytochemical Study"

3. Type of Contract:

Support Agreement Cost Type Other

4. Amount of AEC Funds To Be Obligated by This CA: \$24,409.00

5. AEC Percentage of Est. Total Cost To Be Shown by This CA: 73%

6. Description of Other Changes To Be Covered by This CA:

New contract to provide for the performance of research to be completed during the period November 15, 1969 - November 14, 1970. AEC support ceiling will be as indicated in Items 4 and 5 above. Title to equipment shall vest in the contractor under authority of the AEC Act of 1954 since the contractor's contribution is expected to equal or exceed the value of the equipment. The project will be under the direction of Dr. Jerry D. Berlin.

7. Authority:

Form AEC-481 (Cont. Authr.) from J. R. Tattor dated November 4, 1969.

OLE: JDB

Herman M. Roth, Director
Laboratory and University Division

OLE
12/1/69

CONTRACTS-4002 (Texas)

1034893

APPENDIX "A"

For the Contract Period November 15, 1969, through November 14, 1970.

A-I RESEARCH TO BE PERFORMED BY CONTRACTOR

The Contractor will conduct microscopic (electron) and cytochemical studies on biopsied tissues from normal human testes which have received x-irradiation at doses of 200 rad and 600 rad. Samples will be obtained from a few minutes to several months after irradiation to define the enzymic localizations that take place during a number of phases, i.e., spermatogonial degeneration, and the recovery processes when the seminiferous tubules are repopulated with the spermatogenic cell series. Effects of irradiation on structure and function will be a principal consideration.

The Principal Investigator expects to devote approximately 20% of time for nine months and 100% of time for 1 1/2 months to the work.

A-II WAYS AND MEANS OF PERFORMANCE(a) Items Included in Total Estimated Cost:

(1) <u>Salaries and Wages:</u>	\$12,350.00
Dr. Jerry Berlin, Principal Investigator 2 Graduate Students	
(2) <u>Fringe Benefits:</u>	593.00
(3) <u>Supplies and Materials:</u>	3,038.00
(4) <u>Equipment:</u>	10,000.00
Ultramicrotome and Light Microscope	
(5) <u>Communications:</u>	50.00
(6) <u>Publication Costs:</u>	400.00
(7) <u>Travel:</u>	1,000.00
(8) <u>Electron Microscope Service Contract:</u>	1,600.00
(9) <u>Indirect Charges (36.74% of Salaries and Wages):</u>	4,538.00

(b) Items Significant to the Performance of This Contract,
But Excluded From Computation of Total Cost and From
Consideration in Proportioning Costs:

None

(c) Time or Effort of Principal Investigator to be Contributed
by Contractor:

Cone under this paragraph

A-III The total estimated cost of items under A-II (a) above for the contract period stated in this Appendix "A" is \$33,569.00; the Commission will pay 73% of the actual costs of these items incurred during the contract period stated in this Appendix "A", subject to the provisions of Article III and Article B-XXVII. The estimated AEC support cost for the contract period stated in this Appendix "A" is \$24,409.00.

NOV 4 1969

Dr. Jerry D. Berlin
Department of Biology
Texas Technological College
Lubbock, Texas 79409

Dear Dr. Berlin:

I am pleased to inform you that Atomic Energy Commission contribution toward support of your project, "The Effects of X-Irradiation on the Human Testis: An Electron Microscopic Cytochemical Study," has been approved but at less than the requested level.

The negotiation of the contract, including financial detail, will be handled by our Oak Ridge Operations Office and you may expect to hear from a representative of that office shortly. Responsibility for the technical and scientific administration will, of course, remain with the Division of Biology and Medicine in Washington.

We wish you every success in your work.

Sincerely,

Frank T. Brooks, D.V.M.
Medical Research Branch
Division of Biology and Medicine



cc: OROO

A10835

1034897

1. First-Year Budget (From November 15, 1969 to November 14, 1970)

	<u>Estimated Requirements</u>	
	<u>1st Year</u>	
	<u>Texas Tech University</u>	<u>Atomic Energy Commission</u>
1. Salaries and Wages		
a. Principal Investigator, J. Berlin, Assoc. Prof., \$1633 per month (Full-time for 1 1/2 months) (1/5 time for 9 months)	\$ 2,939.00	\$ 2,450.00
b. Graduate Assistants		
Graduate Assist. (1/2 time for 9 months)		\$ 2,500.00
Graduate Assist. (1/2 time for 9 months)		2,700.00
Graduate Assist. (Full-time for 3 months) Master's Candidate		861.00
Graduate Assist. (Full-time for 3 months) Doctoral Candidate		900.00
c. Fringe Benefits (Social Security, etc. at 4.8% of above salaries)	141.00	452.00
2. Supplies and Materials		
a. Chemicals		600.00
b. Glassware		400.00
c. Electron Microscope Plates, other Materials		688.00
d. Other Photographic Supplies		400.00
e. Diamond Knives (two)		900.00
f. Office Supplies and Miscellaneous		50.00
3. Equipment		
a. Ultramicrotome		5,000.00
b. Light Microscope	5,000.00	
4. Publication Costs		400.00
5. Communication		50.00
6. Travel		
a. To Scientific Meeting		400.00
b. To Seattle, Washington & Salem, Oregon		600.00
7. Hitachi Electron Microscope Service Contract		1,600.00
8. Indirect Charges 36.74% of salaries and wages in accordance with policy established with DOD TDS 66-67	1,080.00	3,458.00

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CONTRACTS - 4002 (Texas)
A11206

NOV 19 1969

TOTAL PROJECT \$33,569.00
 Percentage and Amount Contributed \$ 9,160.00
 by Texas Tech University-27.3%
 Percentage and Amount Requested \$24,409.00
 from the AEC-72.7%

J. Second-Year Budget (From November 15, 1970 to November 14, 1971)

	<u>Estimated Requirements</u>	
	<u>Texas Tech University</u>	<u>Atomic Energy Commission</u>
1. Salaries and Wages		
a. Principal Investigator, J. Berlin, Assoc. Prof., \$1715 per month (Full-time for 1-1/2 months) (1/5 time for 9 months)	\$ 3,087.00	\$ 2,573.00
b. Graduate Assistants		
Graduate Assist. (1/2 time for 9 months)		2,500.00
Graduate Assist. (1/2 time for 9 months)		2,700.00
Graduate Assist. (Full-time for 3 months)		861.00
Graduate Assist. (Full-time for 3 months)		900.00
c. Fringe Benefits (Social Security, etc. at 4.8% of above salaries	148.00	458.00
2. Supplies and Materials		
a. Chemicals		600.00
b. Glassware		300.00
c. Electron Microscope Plates, other Materials		600.00
d. Other Photographic Supplies		400.00
e. Resharpen diamond knives		200.00
f. Office supplies and miscellaneous		50.00
3. None Requested for 2nd Year		
4. Publication Costs		600.00
5. Communication		50.00
6. Travel		
a. To Scientific Meeting		400.00
b. To Seattle, Washington & Salem, Oregon		600.00
7. Hitachi Electron Microscope Service Contract		1,600.00
8. Indirect Charges 36.74% of salaries and wages in accordance with policy established with DOD TDS 66-67	1,134.00	3,503.00

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TOTAL PROJECT COST	\$23,264.00
Percentage and Amount Contributed by Texas Tech University-18.8%	\$ 4,369.00
Percentage and Amount Requested from the AEC-81.2%	\$18,895.00

XX

USAEC
C. S. SHOUP, CHIEF
RESEARCH CONTRACTS BRANCH
LABORATORY AND UNIVERSITY DIVISION
OAK RIDGE, TENNESSEE

DR. JERRY D. BERLIN
ASSOCIATE PROFESSOR
DEPARTMENT OF BIOLOGY
TEXAS TECHNOLOGICAL COLLEGE
LUBBOCK, TEXAS

INFO: MR. M. L. PENNINGTON
VICE PRESIDENT FOR BUSINESS
AFFAIRS
TEXAS TECH COLLEGE
LUBBOCK, TEXAS

XX

ORIGINAL SIGNED BY
C. S. SHOUP
C. S. SHOUP, CHIEF

NOV 7 1969

RECEIVED AUTHORIZATION FROM AEC HQ TO NEGOTIATE AND ADMINISTER A SPECIAL RESEARCH SUPPORT AGREEMENT WITH TEXAS TECHNOLOGICAL COLLEGE COVERING YOUR PROJECT ON THE EFFECTS OF X-IRRADIATION ON THE HUMAN TESTIS: "AN ELECTRON MICROSCOPIC CYTOCHEMICAL STUDY". AEC SUPPORT CEILING APPROVED IN AN AMOUNT NOT TO EXCEED \$24,000 FOR A PERIOD OF ONE YEAR. UNLESS COLLEGE WILL ACCEPT AEC SUPPORT OF \$24,000 TO AN ESTIMATED TOTAL PROJECT COST OF \$41,575 AS OUTLINED IN PROPOSAL, PLEASE SUBMIT A REVISED BUDGET BASED ON REDUCED AEC SUPPORT AND SUPPORT TO BE PROVIDED BY COLLEGE. INFO SUBMITTED SHOULD BE ENDORSED BY APPROPRIATE CONTRACTING OFFICIAL OF COLLEGE. WE PLAN TO HAVE PROJECT COMMENCE ON NOVEMBER 15, 1969, AND EXPIRE ON NOVEMBER 14, 1970. IF COMMENCEMENT DATE IS NOT SATISFACTORY, PLEASE INDICATE DESIRED DATE

CONTRACT WILL BEAR NUMBER AT-(40-1)-4002.

OLE:JDB -45

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OFFICE Adm. SER. Br. JDB:slc SURNAME DATE 11-7-69	C. S. SHOUP 11/2/69	CONTRACTS - 4002 (Tel)
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PROPOSAL FOR
THE UNITED STATES ATOMIC ENERGY COMMISSION
SUPPORT OF RESEARCH

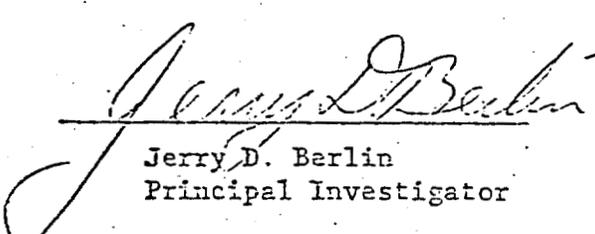
Project Title: The Effects of X-irradiation on the Human Testis:
An Electron Microscopic Cytochemical Study

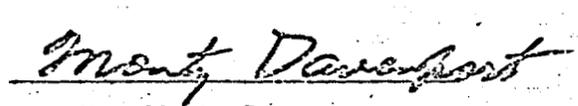
Desired Starting Date: September 1, 1969

Institution: Texas Technological College
Lubbock, Texas 79409

Principal Investigator: Jerry D. Berlin
Associate Professor
Department of Biology

Authentication:


Jerry D. Berlin
Principal Investigator


Dr. Monty Davenport
Associate Vice President
for Research

1034902

THIS IS TO CERTIFY That the equipment, specifically
an ultramicrotome, requested for purchase in the Atomic Energy
Commission Research Proposal entitled "The Effects of X-irradiation
on the Human Testis: An Electron Microscopic Cytochemical Study"
is not reasonably available for use on this project at Texas
Technological College.

TEXAS TECHNOLOGICAL COLLEGE

Monty Davenport

Monty E. Davenport
Associate Vice President for
Research and Special Programs

1034903

PROJECT ABSTRACT

Control and X-irradiated (200 and 600r) testes of normal men will be subjected to an electron microscopic cytochemical examination. Samples will be obtained from a few minutes to several months after irradiation to define enzymic localizations that occur during (1) spermatogonial degeneration, i.e., early radiation effects, and (2) the recovery process when the seminiferous tubules are repopulated with the spermatogenic cell series. Information derived from this study should provide knowledge of the effects of X-radiation on the structure and function of these important cells.

1034904

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A. Scientific Background:

The material for this study will be obtained through the courtesy of Dr. Carl Heller, Director, Division of Reproductive Physiology, Pacific Northwest Research Foundation, Seattle, Washington, who has kindly consented to supply the Principal Investigator with human spermatogenic tissue from volunteer inmates residing in the Oregon State Prison, Salem, Oregon. Dr. Heller's program projections are such that the material required for this study will be available during the normal course of his work. Thus, experimental manipulation of human subjects will not be duplicated for the proposed study. In fact, we view the proposed work as an effort to obtain the maximum amount of scientific information from this rare and unique experimental material.

The Principal Investigator of the proposed study previously cooperated with Dr. Heller on an electron microscopic study of human spermatogenic tissue. In reality, the proposed work is a continuation and extension of that study. We have identified four distinct types of human spermatogonia by fine structural criteria, whereas previously only three spermatogonial types were recognized (see Appendices A and B). Also pertinent to the proposed work was an initial study made in the area of electron microscopic cytochemistry. Although we were not able to complete those studies before the Principal Investigator moved to Texas Technological College, we were successful in processing tissue obtained from the prison in a laboratory removed from the prison by approximately 350 miles. The same techniques that we have previously used for remote sample preparation will be used without modification in the proposed study.

It is well known that the testis is an extremely radiosensitive tissue. In the mouse, Oakberg (1955a, 1955b) has shown that radiation-induced sterility is the result of spermatogonial degeneration and is not due to an inhibition of mitosis. Heller et al. (1965) have shown spermatogonial damage in human testes as early as three hours postexposure. These same workers reported complete denuding of the germinal epithelium in man at 200r and above and found moderate oligospermia at doses as low as 15r. A decrease in sperm count was not found until 46 days postexposure, which is the time required for preleptotene spermatocytes to develop into mature sperm in man. The most advanced spermatogonia gives rise to the preleptotene spermatocyte, therefore, it appears that spermatogonial damage is also the major factor in the radiosensitivity of the human testis.

Light microscopic investigations of the human testis are numerous and references to this area may be found in the excellent work of Clermont (1963, 1966a, 1966b) and Heller and Clermont (1964). Brief descriptions of human spermatogonia, based on light microscopic examinations of paraffin and Epon embedded sections are included in Appendix B.

A number of electron microscopic studies have been performed on the human testis (Fawcett and Burgos, 1960; Villar, Perez del Cerro, and Mancini, 1962; Bawa, 1963; Leason 1966; Nagano, 1962, 1966; de Kretser, 1967a, 1967b; Smith Leason, and Bunge, 1967; Smith, Leason, Bunge, and Anderson, 1967; Yasuzumi, Nakai, Tsubo, Yasuda, and Sugioka, 1967; Tres and Solari, 1968; Berlin, 1968a,

and Berlin and Adec, 1969). In general these studies have utilized abnormal or unspecified tissue. Also, the majority of the above investigations have described the fine structure of the interstitial cells, Sertoli cells, or spermatids. The ultrastructure of the spermatogonia, which are the most radiosensitive cells, has until recently received very little attention. In addition to our work, the fine structure of the germinal cells of normal men has been reported only in abstract form (Villar and Paulsen, 1967).

The effects of irradiation on the subcellular morphology of the human testis have not been reported. I previously obtained human testicular tissue one year after an exposure of 200r. The only cells observed within the seminiferous tubules in a brief survey of this material were Sertoli cells. The question raised by this observation pertains to the cell type that gives rise to spermatogonia during the recovery process to effect repopulation of the spermatogenic cell series. At the present time the identification of these "precursor" cells is strictly a matter for speculation. As pointed out in the preprint in Appendix B, irradiation studies represent a means by which the most primitive spermatogonium can be identified.

Electron microscopic cytochemical observations on human testes are somewhat limited. Yasuzumi et al. (1967) suggested that adenosine triphosphatase activity was localized in the nuclear pores of the Leydig cells of aged human testes. Adenosine triphosphatase activity has been localized in a previously undescribed filamentous body that occurs in certain types of human spermatogonia (See Figure 13, Appendix B). This technique has been used with convincing results to localize a number of enzymes in rat and guinea pig testes (Tice and Barnett, 1963; Gordon and Barnett, 1967; Reddy and Svoboda, 1967; Frank and Christensen, 1968; and Posalaki, Szabo, Bacsi, and Okos, 1968). In addition, Hugon and Borgers (1966) utilized electron microscopic cytochemistry to demonstrate a transitional increase in acid phosphatase activity in the Sertoli cells of irradiated mice. The Principal Investigator has used the technique to demonstrate an immediate radiation response in intestinal epithelial cells (Berlin, 1968b) and also as a means to assay cellular damage resulting from heavy metal toxicity in hepatocytes (Appendix C). It is doubtful that any other technique is as capable of assaying immediate biological responses to radiation as is electron microscopic cytochemistry.

The cytochemical localization of phosphatase enzymes presents a method whereby function may be correlated with structure. The phosphatases are involved in many, if not all, basic cellular processes, e.g., they play a role in digestion, intracellular transport, intercellular transport, biosynthesis, etc. Clearly, it is important to have an ultrastructural characterization of these important enzymes under control and irradiated conditions in man. On the one hand, this information would contribute to a better description of radiation damage. On the other hand, such knowledge might yield information enabling a better interpretation to be made about cellular properties and activities of the living testis.

One of the reasons for working with man and not with another species in the proposed study is because human testes are sufficiently different morphologically from other species reported in the literature as to cast doubts on any attempt at extrapolation. For example, man alone has well defined crystalloid inclusions

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in the Leydig cells and in the Sertoli cells. Man alone has a filamentous body in the spermatogonia. Man has only four recognizable types of spermatogonia. Man has no equivalent to the "wave of the seminiferous epithelium" described in rat and other mammals. In a different vein, 800 r hardly affects the bull, whereas 15 r has a marked effect on man. For these and many other reasons, extrapolation from experiments with other animals to man has a number of built-in hazards and is fraught with difficulty. Thus, if one is interested in resolving problems in human tissues one must look at man as there is no suitable substitute. Nevertheless, it should be emphasized that the work outlined in this proposal will not require additional manipulation of human subjects, but will take advantage of available experimental tissue.

B. Scientific Scope:

The objective of the proposed study is to define changes in ultrastructure and enzyme localization that occur after X-irradiation of human testes. Emphasis will be placed on the early effects of irradiation and especially on spermatogonial degeneration. Obviously, it will be necessary to determine enzyme localizations in control tissues as this has not been accomplished in the human. In addition, the recovery process will also be examined and it is anticipated that we will be able to determine the cell type that repopulates the spermatogenic cell line during the recovery period. We further anticipate that enzymic recovery occurs, perhaps prior to cellular repopulation, and that we will be able to establish a pattern for this process. This work is designed to extend to the subcellular level our present knowledge about the effects of X-irradiation on the human testis. The Principal Investigator has no knowledge of any comparable work in progress in any other laboratory.

C. Experimental Design:

Control and irradiated human testicular tissue will be supplied by Dr. Carl Heller, Pacific Northwest Research Foundation, Seattle, Washington. Tissue samples will be available in a variety of times following exposure to 200 and 600 r X-radiation. We will be particularly interested in immediate effects (15 minutes to 6 hours postexposure) and in selected times during the recovery period. At the early times we will follow spermatogonial degeneration and at the later times we will determine the sequence of subcellular events leading to repopulation.

Biopsied testicular material will be initially fixed at the prison by a technician from Dr. Carl Heller's laboratory in Seattle. It is deemed desirable for the Principal Investigator to make two trips a year to Seattle to direct and consult on prison procedures. These trips will also be valuable for planning our experiments to coincide with Dr. Heller's ongoing program. To obtain properly fixed material the technician will split the biopsy into two parts. One part being fixed in freshly prepared 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, 0°C) and the other part in freshly prepared cold 4% formaldehyde in 0.1 M cacodylate buffer containing 1% CaCl₂ (different fixatives being required to retain activity of different enzymes). Immediately after placing the tissue into the appropriate fixative the samples will be cut into 1 X 2 X 5 to 10 mm strips

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to ensure adequate penetration of the fixative. The major precaution for this step is that the surface of the tissue not be allowed to dry, therefore, the tissue will remain submerged in the chilled fixative during the cutting procedures. The tissue will remain in the initial fixative for 55 minutes, but no longer than one hour (longer times in the initial fixative inactivates several of the enzymes for which we will be subsequently assaying). At the end of the initial fixation period, the tissue will be transferred to cold 0.1 M cacodylate buffer (containing 5% sucrose when required, depending upon the enzymes to be localized), packed in ice, wrapped in a brilliant red paper, and shipped by air to the Principal Investigator at Texas Technological College. The schedules will be prearranged and the Principal Investigator will meet the incoming plane and retrieve the sample. We have personally found that wrapping the samples in brilliant red paper has two advantages. First, they appear to receive more intimate care from airlines personnel and second, they are readily identifiable and easily retrievable. The above procedures have been described in detail because of the necessity of utilizing technical help at the prison (see K. Budgetary Addendum).

We previously found that the above procedure routinely gave satisfactory results. The major requirement for transporting samples is that they be kept chilled. The method of packing that we previously used will keep the tissue satisfactorily chilled for at least 24 hours. This is ample time for its shipment to the Principal Investigator's laboratory which will require a maximum of eight hours. Transport of the tissue at this particular step does not damage the tissue and, in fact, a buffer wash of 18 to 24 hours at this interval is routine procedure in specimen preparation for electron microscopic cytochemistry (Smith and Farquhar, 1965).

When received at Texas Technological College the tissues will be further processed for the localization of acid phosphatase, various nucleoside di- and triphosphatases, and thiamine pyrophosphatase. The preparations will be made in the routine manner for electron microscopic cytochemistry with a tissue sectioner being employed to obtain 40 micron sections. These sections will be further washed in chilled 0.1 M cacodylate buffer prior to their incubation in medium containing appropriate substrates. During incubation the sections will be monitored where possible at the light microscope level to ensure proper incubation times. After incubation the sections will be given a buffer wash, fixed in osmium tetroxide, dehydrated in ethanol, and embedded in Epon. After polymerization the tissues will be sectioned for both light and electron microscopic examination. An ultramicrotome is required for thin sectioning, i.e., to obtain sections that are 300 to 500 Angstroms thick. This same instrument will be used to obtain 1 micron thick sections for light microscopy. The thick sections will be stained with azure II and methylene blue (Richardson et al., 1960) and by a modified PAS stain (Maser, 1968). Light microscopy will be used for orientation and as a survey aid to the electron microscopic investigation. A possibility that might arise is that the six cell associations found in human testes (Clermont, 1963) will possess different patterns of enzymic localizations. The light microscope will be an indispensable instrument for the study because the cell associations are virtually impossible to identify by electron microscopy due to the small amount of tissue that can be observed. Thus light microscopy will be used to identify the cellular associations on sections adjacent to those used for the ultrastructural study so that we may examine the possible influence of the cell associations on enzymic localizations. Thin sections obtained with the ultramicrotome will be examined

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via the electron microscope either unstained or stained by a modified uranyl acetate-lead citrate stain (Adee and Berlin, ca. 1960). Unstained sections will be used to avoid any possibility of electron dense staining contaminants that might be confused with the enzymic reaction product.

This project will have an important role in the development of the Principal Investigator's graduate program at Texas Technological College. The primary reasons the author recently accepted his present position were the challenges inherent in establishing an electron microscope laboratory in our new facility and in developing a graduate program in Cell Biology. The proposed study will substantially enhance our graduate program by providing training for graduate students with a unique tissue and with the technique of electron microscopic cytochemistry.

D. Scientific Personnel:

1. Principal Investigator: Jerry D. Berlin, Ph.D.

Present Position: Associate Professor

Fraction of Time of Project:

Academic Year: 20%

Summer Months: 50%

Social Security Number: [REDACTED]

Scientific Experience:

Dr. Berlin was a Senior Research Scientist in the Biology Department at Hanford Laboratories (operated by Battelle Memorial Institute for the Atomic Energy Commission) Richland, Washington for four years (September, 1964 to August, 1968). During that time he directed the Electron Microscope Laboratory and obtained considerable experience with the subcellular effects of radiation injury. During the tenure of that employment an electron microscopic study of human spermatogenic tissue was initiated with Dr. Carl Heller, Director of Reproductive Physiology, Pacific Northwest Research Foundation, Seattle, Washington. The proposed study is an extension of that study. Presently Dr. Berlin is initiating a graduate program at Texas Technological College.

Publications:

Bowen, C.C., G. A. Peyton, and J. D. Berlin. 1963. The fine structure of the host-parasite interface of some obligate fungal parasites. *Amer. J. Botany* 50: 624-625.

Berlin, J. D. and C. C. Bowen. 1964. The host-parasite interface of Albugo candida on Raphanus sativus. *Amer. J. Botany* 51: 445-452.

Berlin, J. D. and C. C. Bowen. 1964. Centrioles in the fungus Albugo candida. *Amer. J. Botany* 51:650-652.

Berlin, J. D. and C. C. Bowen. 1965. Mitosis and zoospore formation in Albugo. *Amer. J. Botany* 52: 613.

E. Other Personnel:

Technician (to be named).

Fraction of Time on Project: 100%

Justification: A technician will be needed for this project to prepare solutions, print and dry electron micrographs and help with sample preparation (including incubation, fixation in osmium tetroxide, dehydration, embedding and sectioning). By performing routine laboratory tasks the technician will free the investigator's time for the more critical microscopic work.

F. Other Financial Assistance:

From non-university sources for this project: None

From non-university sources on unrelated projects:

The Principal Investigator has a \$5,000 grant from the National Cotton Council for unrelated work that will terminate December 31, 1969.

G. Facilities:

The Department of Biology at Texas Technological College, where the work outlined in this proposal will be performed, has under construction a new Biology Building that is scheduled for completion by September 1, 1969. We are requesting that this project be initiated simultaneously with that date. This facility will include an Electron Microscope Laboratory of approximately 3600 square feet of floor space. Of this, approximately 1500 square feet is devoted to a teaching laboratory that will contain a Hitachi model HS-8 electron microscope. This teaching complex has an electron microscope room, a plate and print darkroom, a general preparation room and a teaching laboratory. The remaining 2100 square feet of floor space has been allocated for a research complex that will contain a Hitachi model HU-11E-1 electron microscope and will be available for this study. The research complex has a general preparation room, cold room, print darkroom, microtomy room, electron microscope room, plate dark room, a room to be used specifically for cytochemistry, and office space for the Principal Investigator. Separate office space will be provided for the Graduate Assistants. Monies are available for equipping this complex with two electron microscopes, one microtome, a tissue sectioner, etc. The electron microscopes have already been ordered and are to be installed by August 1, 1969. However, the one microtome will be placed in the teaching laboratory for student training and will not be available for this study. For this reason, we are requesting, as a line item in the budget, money for an ultramicrotome that would be necessary for this study. Texas Technological College will match the funds being requested from the Atomic Energy Commission for an ultramicrotome and these matching funds will be used to obtain a research light microscope that is necessary for this project. All other equipment required for the successful completion of this study will be provided by Texas Technological College.

I. First Year Budget (From September 1, 1969 to August 31, 1970)

	<u>Estimated Requirements</u>	
	1st Year	
	Texas Technological College	Atomic Energy Commission
1. Salaries and Wages		
a. Principal Investigator, J. Berlin, Assoc. Prof., \$1633 per month (Full time for 1 1/2 months) (1/5 time for 9 months)	\$ 2939.00	\$ 2450.00
b. Graduate Assistants		
Graduate Assist. (1/2 time for 9 months)		2500.00
Graduate Assist. (1/2 time for 9 months)		2700.00
Graduate Assist. (Full time for 3 months) Master's Candidate		861.00
Graduate Assist. (Full time for 3 months) Doctoral Candidate		900.00
c. Technician (to be named)		6000.00
d. Fringe benefits (Social security, etc. at 4.8% of above salaries)	141.00	740.00
2. Supplies and materials		
a. Chemicals		600.00
b. Glassware		400.00
c. Electron microscope plates, other materials		600.00
d. Other photographic supplies		400.00
e. Diamond Knives (two)		900.00
f. Office supplies and miscellaneous		50.00
3. Equipment		
a. Ultramicrotome		5000.00
b. Light microscope	5000.00	
4. Publication costs		400.00
5. Communication		50.00
6. Travel		
a. To scientific meeting		400.00
b. To Seattle, Washington and Salem, Oregon		600.00
7. Hitachi electron microscope service contract		1600.00
8. Indirect charges 34.57% of salaries and wages in accordance with policy established with DOD TDS 66-67	1016.00	5328.00

1034912

TOTAL PROJECT COST \$ 41,575.00
 Percentage and Amount Contributed
 by Texas Technological College 21.9% \$ 9,096.00
 Percentage and Amount Request
 from the AEC 78.1% \$ 32,479.00

J. Second Year Budget (From September 1, 1970 to August 31, 1971)

	<u>Estimated Requirements</u>	
	2nd Year	
	Texas Technological College	Atomic Energy Commission
1. Salaries and Wages		
a. Principal Investigator, J. Berlin, Assoc. Prof., \$1715 per month (Full time for 1 1/2 months) (1/5 time for 9 months)	\$ 3087.00	\$ 2573.00
b. Graduate Assistants		
Graduate Assist. (1/2 time for 9 months)		2500.00
Graduate Assist. (1/2 time for 9 months)		2700.00
Graduate Assist. (Full time for 3 months)		861.00
Graduate Assist. (Full time for 3 months)		900.00
c. Technician (Full time for 12 months)		6300.00
d. Fringe benefits (Social Security, etc. at 4.8% of above salaries)	148.00	760.00
2. Supplies and materials		
a. Chemicals		600.00
b. Glassware		300.00
c. Electron microscope plates, other materials		600.00
d. Other photographic supplies		400.00
e. Resharpen diamond knives		200.00
f. Office supplies and miscellaneous		50.00
3. None requested for 2nd year)		
4. Publication costs		600.00
5. Communication		50.00
6. Travel		
a. To scientific meeting		400.00
b. To Seattle, Washington and Salem, Oregon		600.00

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7. Hitachi electron microscope service contract		1600.00
8. Indirect Charges 34.57% of salaries and wages in accordance with policy established with DOD TDS 66-67	1067.00	5474.00

TOTAL PROJECT COST \$ 31,770

Percentage and Amount Contributed
by Texas Technological College 13.5% \$ 4,302

Percentage and Amount Request
from the AEC 86.5% \$ 27,468

1034914

K. Budgetary Addendum

In relying upon prison help to do the initial portion of our preparations, we have previously encountered a number of difficulties. For example, after months of exasperation we finally traced one problem to the fact that the inmate technician had placed the tissue in buffer to which fixative had not been added. Our cytochemical work involves more complex procedures and we feel that prison help is technically incapable of satisfactory performance. Because of the nature of this work, the rarity of the material involved, and the amount of time consumed in an experiment a request will be made by Dr. C. Heller in his Atomic Energy Commission Proposal to have a technician from his Seattle laboratory travel to the prison in Salem, Oregon and perform our initial fixation. This procedural technicality in the budget conforms to advice previously solicited (letter dated April 25, 1969) and received (phone call April 30, 1969) by the Principal Investigator from the Atomic Energy Commission.

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2. Graduate Assistant: James Ramsey, M.S.
 Graduate Student
 Social Security Number: [REDACTED]
 Fraction of Time on Project:
 Academic Year: 50%
 Summer: 100%
 Scientific Experience: Mr. Ramsey will receive his Master's degree in June; his thesis involves a biochemical problem and is being prepared for publication.
 Publications: None

3. Graduate Assistant: John J. Vollet, III, B.S.
 Graduate Student
 Social Security Number: [REDACTED]
 Fraction of Time on Project:
 Academic Year: 50%
 Summer: 100%
 Scientific Experience: None
 Publications: None

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APPENDIX A

ABSTRACT OF PAPER PRESENTED TO
THE PACIFIC COAST FERTILITY SOCIETY 16th ANNUAL MEETINGS
October 31-November 3, 1968, Palm Springs, California

1034921

1967

Pacific Coast Fertility Society
Ann. Mtg. Oct 31 - Nov 3, 1968
Palm Springs Calif

THE FINE STRUCTURE OF HUMAN SPERMATOGONIA

Jerry D. Berlin, Mavis J. Rowley and Carl G. Haller

Battelle Memorial Institute, Richland, Washington

Pacific Northwest Research Foundation, Seattle, Washington

Prior study of the human testes using the light microscope has shown three types of spermatogonia. Electron microscopic examination of normal human testes has now revealed the existence of four distinct types of spermatogonia. These are the dark type A1 (Ad-1), the dark type A2 (Ad-2), the pale type A (Ap), and the type B (B) spermatogonia. With the addition of a fourth type it now becomes possible to distinguish which cell is the most primitive and the sequence of their development. The subcellular criteria used in distinguishing between these four spermatogonial types include the density of the nucleoplasm, the type of nucleolus and its placement within the nucleus, the structure of the mitochondrial cristae, the association of the endoplasmic reticulum with the mitochondria, the amount of glycogen present within the cell, and the presence of previously undescribed filamentous structures in the cytoplasm of the Ad-1 and Ad-2 spermatogonia. Each spermatogonium is in contact with the basal lamina; the amount of contact progressively decreases from the Ad-1, a small flat cell lying parallel to the basal lamina, through the Ad-2 and Ap to the B spermatogonia, the latter being a pear-shaped cell with its long axis perpendicular to the basal lamina. In addition to describing the fine structure of the four types of spermatogonia we will discuss the possible roles the various structures play in spermatogonial development.

1034922

APPENDIX B

PREPRINT OF PAPER SUBMITTED TO THE AMERICAN JOURNAL OF ANATOMY

1034923

THE ULTRASTRUCTURE OF FOUR TYPES OF HUMAN SPERMATOGONIA

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The Ford Foundation.

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INTRODUCTION

Spermatogenesis is a continuous process that starts prior to maturity and persists for most of the human life. The cells from which all subsequent generations of germinal cells arise are called spermatogonia. These cells exist in the human testes in regular numbers dividing mitotically to form the more differentiated germinal elements. They lie against the basal lamina of the seminiferous tubule and together with the Sertoli cells form a cellular layer along the basement lamina of the tubule.

The identification of the types of spermatogonia and the tentative outline of their sequential development in man was begun in the recent investigations on the normal human testes by Clermont (1963) and Heller and Clermont (1964). These studies, using light microscopy, identified three spermatogonial types in man; the A dark (Ad), the A pale (Ap) and the B spermatogonia. The main morphological features used in distinguishing between these three types of spermatogonia at the light microscope level are the shape and staining characteristics of the nucleus, the placement of the nucleolus and the presence or absence of glycogen in the cytoplasm. The Ad spermatogonium was suggested to be the least differentiated morphologically. The type B spermatogonium was considered to be the most differentiated of the three cells

for two reasons: 1) it occurs in only four of the six cell associations and 2) daughter cells formed by its division are identified as spermatocytes. Both the Ad and the Ap spermatogonia are found in all six cell associations and are flattened against the basal lamina of the seminiferous tubule.

Roosen-Runge and Barlow (1953) carefully studied spermatogonial size and related their data to that of earlier workers. Although their conclusions were based on an erroneous premise (no organization in the human seminiferous epithelium), their measurements reflect the true variation in spermatogonial size. Clermont (1966), through painstaking mapping of the seminiferous tubules, worked out a model of spermatogonial development. Mancini (1965) made an elaborate attempt to classify spermatogonia in cryptorchid and scrotal human testis.

Electron microscopy of the human testis has, until recently, been restricted to observations of abnormal material (Smith, Leeson, Bunge, and Anderson, 1957; Fawcett and Burgos, 1960; de Kretser, 1967a; Leeson, 1966; and Smith, Leeson and Bunge, 1967), tissue of unspecified origin or normalcy (Nagano, 1956; Vilar, Perez del Carro and Mancini, 1962; Nagano, 1962) and tissue of normal or aging human testes (de Kretser, 1967b; Vilar and Paulson, 1967; Yasuzumi, Nskai, Tsubo, Yasuda and Sugioka, 1967; Tres and Solari, 1968). The majority of these investigations describe the fine structure of the interstitial cells, Sertoli cells or spermatids and only an occasional and incidental electron micrograph of a human

spermatogonium has been published. Vilar and Paulsen (1957) have published an abstract on the fine structure of the human germinal cells. These workers reported two types of spermatogonia lying on the basal lamina and a third type surrounded entirely by Sertoli cell cytoplasm.

Two difficulties have contributed to the paucity of information about human spermatogonia; obtaining the proper material, and identifying the germinal cells. We have overcome the first difficulty by obtaining biopsy specimens from testes of normal healthy young adults, the second was overcome by using three separate morphological studies to insure accurate identification of the spermatogonial types. First, we examined paraffin embedded tissues with the light microscope in the classical manner to identify the three recognized types of spermatogonia. Second, we studied Epon embedded tissues sectioned at micron or sub-micron thicknesses by light microscopy and identified the spermatogonial types by comparing the results with the paraffin embedded material. Third, electron microscopic examination of thin sections adjacent to sections used for light microscopic examination was used to determine the ultrastructural characteristics. In this way we felt assured that the cells we called Ad, Ap and B spermatogonia in paraffin sections corresponded to the cells we term Ad, Ap and B spermatogonia in Epon sections. studied with either the light or electron microscope. The identifying characteristics of human spermatogonia based on these comparative studies are herein described.

MATERIALS AND METHODS

Biopsy specimens from testes of four normal healthy adults that were inmate volunteers of the Oregon State Penitentiary were used in these studies. The men ranged in age from 29 to 50 years. None had a history of disease of the reproductive system. Individuals were selected for this study on the basis of a series of normal sperm counts and normal urinary outputs of gonadotropins and testosterone. Bilateral testicular biopsies were performed as previously described (Rowley and Heller, 1966) and the biopsy was immediately transferred to either Cleland's fixative (Rowley and Heller, 1966) for subsequent paraffin embedding or to glutaraldehyde fixative for Epon embedding. Tissues embedded in Epon were trimmed to blocks of 1 mm³ during fixation. The glutaraldehyde fixative consisted of either 3% purified glutaraldehyde in 0.067 M phosphate buffer at pH 7.3 or 1% purified glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3. Tissues fixed in glutaraldehyde were post-fixed in OsO₄ (Millonig, 1961), dehydrated in ethanol, and embedded in Epon (Luft, 1961). Paraffin embedded material was sectioned at 4 μ and stained with iron hematoxylin-eosin. Epon embedded material was sectioned at 1 μ or less and stained with Richardson's stain (Richardson, et al, 1960). For electron microscopy, gray-silver sections of the Epon embedded tissue were stained with uranyl acetate and post-stained with lead citrate (Reynolds, 1963). ATPase was localized by a modification (Burstone, 1962) of the

method of Wachstein and Meisel (1957). Characteristics of the testicular material of the four subjects were similar although slight variation was found in the depth of staining obtained in thin Epon sections.

RESULTS

The terminology A "dark" and A "pale" derives from the descriptions of Clement (1963). He found that one spermatogonium had a dark nucleus with irregularly staining nucleoplasm (A dark) and that one had pale homogeneous nucleoplasm (A pale). This terminology will continue to be used for the same cells described by ultrastructure in order to enable comparisons between methods.

Three types of spermatogonia were readily identified in paraffin sections, namely, the Ad, the Ap and the B type spermatogonia. These same spermatogonial types were also found in micron-thick Epon sections by light microscopy. Electron microscopic examination revealed these three types of spermatogonia and, in addition, presented sufficient evidence to delineate another type of A spermatogonia, the AL*. Returning to light microscopy we were able to identify the three types of A spermatogonia in Epon sections. We have not found it possible, however, to unequivocally distinguish three types of A spermatogonia in paraffin sections.

* This cell was designated with an L because of its length on the basal lamina and the length of its cylindrical nucleus.

Each spermatogonial cell is in contact with the basal lamina, although the degree of contact progressively decreases from the AL through the Ad and the Ap to the B spermatogonia. That part of the spermatogonial cell surface not in contact with the basal lamina is in juxtaposition either to Sertoli cell surface or to the surface of spermatogenic cells. In each case the plasma membrane of the spermatogonium is separated from the plasma membrane of the adjacent cell by an intercellular space of 130 to 200 Å. Occasionally, pinocytotic vesicles are continuous with the plasma membrane of the spermatogonia. The structure of each type of spermatogonium is described below.

New type A (AL) spermatogonia

As mentioned above it is not possible to identify clearly three types of A spermatogonia in paraffin sections. In micron-thick Epon sections the AL appears as a flattened cell with its longitudinal axis parallel to the basement membrane lamina (Fig. 1). The nucleus is ellipsoidal with its longitudinal axis also parallel to the basement membrane lamina. The margins of the nucleus are frequently irregular and the nucleoplasm is finely granular with some lighter amorphous regions. Nucleoli are always peripherally located in the nucleus. The cytoplasm contains numerous mitochondria that are usually found removed from the basal lamina.

Figure 2 is a survey electron micrograph of an AL type spermatogonium. The amount of searching required to find the AL

spermatogonia suggests that they are not as frequent as the other types of spermatogonia. Electron microscopic examination showed the same flattened cells at the basal lamina. The association of the plasma membrane with the basement lamina occurs over distances of 30 μ or more in one contact area. The elliptical nucleus (Fig. 3) presents a spherical profile in cross-section (Fig. 2). The nuclear envelope is irregular and the nucleoplasm homogeneous (Fig. 3). The nucleoli are found in a peripheral position (Fig. 3). Each nucleolus consists of a diffuse granular zone (Fig. 4). Fibrillar and amorphous zones are not present. Numerous mitochondria are generally positioned at some distance from the basal lamina (Fig. 2), quite frequently associated with the nuclear envelope, and have tubular cristae (Fig. 8). A linear array of two or more mitochondria are often separated by electron-opaque bars or by strands of rough endoplasmic reticulum (Figs. 8 & 9). The bars appear to be structural modifications of the endoplasmic reticulum (Fig. 10). The rough endoplasmic reticulum occurs in single strands. There is very little smooth endoplasmic reticulum. The Golgi complex is typical in appearance, surrounded by vesicles of uniform size and electron density (Fig. 8). Microtubules have been observed in the cytoplasm lying parallel to the nuclear envelope. Coated vesicles are occasionally present (Fig. 8) and paired centrioles have been found. Glycogen occurs primarily as free particles in the cytoplasm (Fig. 2), however, glycogen has been observed also clustered in membrane-limited pockets. A filamentous body is found in the cyto-

plasm (Fig. 11). This spindle-shaped body, having overall dimensions of approximately $0.5 \times 2.0 \mu$, is composed of parallel filaments. The individual filaments are $10 - 15 \mu$ in diameter and an amorphous material is found adhering to the filaments. The filaments are separated from each other by a distance of $20 - 30 \mu$ and electron-opaque spheres of 15μ diameter are found within this space. When cross-sectioned, the filaments have a tubular appearance with an electron-opaque wall that is $3 - 5 \mu$ thick and an electron-transparent lumen that has a diameter of $3 - 5 \mu$ (Fig. 12). The tubules are aligned in linear arrays and when the 15μ electron-opaque spheres are present they appear to be entrapped by a circle of tubules. In tissue incubated for ATPase activity the filamentous bodies show reaction product (Fig. 12).

Another previously undescribed body in the cytoplasm of the AL spermatogonia is a single membrane-limited structure having a diameter of $1 - 2 \mu$ (Fig. 14). These bodies frequently possess internal membranes in a granular matrix. These structures may be lysosomes, however, acid phosphatase incubations were not carried out and we are forced to term these bodies simply lamellar bodies for the present. The lamellar bodies are commonly found only in the AL spermatogonia. Two AL nuclear profiles were observed in a common cytoplasm (Fig. 15).

Dark type A (Ad) spermatogonia

The Ad type spermatogonia in all probability represents the typical Ad spermatogonia described in the light microscopic literature

(Clermont, 1963). In paraffin embedded tissue, this cell type has a spherical or slightly ovoid nucleus that has a fine but deeply stained chromatin granulation. This granulation is so fine that the greater part of the nucleus looks dark with amorphous light areas (Fig. 16). Toward the center of the nucleus, a large pale stained "vacuole" is usually found; a nucleolus being sometimes associated with the "vacuole". Other nucleoli surrounded by a thin rim of clear nucleoplasm are also found in this nucleus. Its cytoplasm is light and unstained in hematoxylin-eosin preparations, but, as shown by the periodic acid-Schiff technique and the amylase treatment, it is usually loaded with glycogen.

In Epon embedded sections viewed by light microscopy the Ad spermatogonia appear as flattened cells closely appressed to the basement membrane lamina (Fig. 17). The nucleus is ellipsoidal to spherical and a "vacuole" may be either peripherally, or more generally, centrally located within the nucleus. The remaining nucleoplasm appears homogeneous and nucleoli are peripherally located. The cytoplasm is lightly stained and mitochondria appear throughout the cytoplasm, although they are predominantly located in the cytoplasm away from the basal lamina.

The Ad spermatogonia appear ovoid but somewhat less elongated than the AL cells (Fig. 18). The nucleus is elliptical to spherical and the nucleoplasm is slightly granular. A region of rarefaction, corresponding to the "vacuole" observed by light microscopy, is usually a prominent feature of these cells (Figs. 18 & 19). The nucleolus may be within this region of rarefaction or it may be

positioned within the granular nucleoplasm. The nucleoli consist of condensed granular regions (Fig. 5). The nucleolus is surrounded by a region of slightly less granular nucleoplasm. Frequently two forms of membraneous specialization occur on the internal side of the nuclear envelope. One specialization is a membraneous layer closely appressed to the internal face of the nuclear envelope (Fig. 20). Because of the appearance of this specialization in cross-section we have termed these structures nuclear envelope stripes or simple "stripes". In a three dimensional view, they may be plaque-like formations. The stripes are composed of at least two membranes and an intermembrane space. In some instances another layer of the stripe has been observed and one finds three electron-transparent regions, all being membrane-limited, with electron-opaque regions occurring on the inner sides of each membrane-limited space. Thus from the cytoplasmic side of the nuclear envelope moving inward there is the outer membrane of the nuclear envelope, the intermembrane space (perinuclear space), the inner membrane of the nuclear envelope, an electron-opaque region, the external membrane of the stripe, an intermembrane region within the stripe, the inner membrane of the stripe, and another electron-opaque region. The different membraneous components of stripes are apparently continuous with the nuclear envelope. A similar region has been described by Fawcett (1966) in intestinal epithelium. The nucleolus is frequently associated with the stripes (Fig. 21).

The second specialization associated with the nuclear envelope is a region surrounded by double membrane (Figs. 22 & 23). Again, because of their appearance we have termed such structures "blebs". When closely appressed to the nuclear envelope the outer membrane of the bleb is separated from the internal membrane of the nuclear envelope by an electron-opaque region (Fig. 22). The nuclear envelope always protrudes into the cytoplasm when associated with this membrane-limited structure (Fig. 23). The contents of the blebs are either particulate in nature, averaging 15 μ in diameter, and resemble the fine particulate material found in the nucleoplasm, or the contents of the membrane-limited body are electron-transparent with fine filaments being present (Fig. 22).

The mitochondria of the Ad spermatogonia appear to have both tubular and plate-like cristae and are frequently found in groups of two or more separated by electron-opaque bars (the bars are similar in structure to the bars joining the mitochondria together in the AL spermatogonia). One or more mitochondria are usually in close proximity to the nuclear envelope (Figs. 17 & 19). Singular strands of rough endoplasmic reticulum are present and are sometimes associated with mitochondria (Fig. 18). Smooth endoplasmic reticulum is present in limited quantities and is associated primarily with the centrosome (Fig. 18). The other cytoplasmic features appear similar to those described for the AL spermatogonia including the presence of filamentous bodies, however, the lamellar bodies appear to be absent in the Ad spermatogonia.

Two Ad nuclei, as denoted by the presence of a region of rarefaction in each nucleus, are shown in a common cytoplasm in Figure 24. Only a bridge of cytoplasm links these two cells suggesting that cytokinesis is nearly, but not quite, terminal. Spindle fibers are visible in the bridge. Karyokinesis, in this case, has obviously been completed.

Pale type A (Ap) spermatogonia

In paraffin sections (Fig. 16) the Ap spermatogonia are characterized by a slightly ovoid nucleus containing a fine pale stained chromatin granulation. They also contain one or two irregularly shaped nucleoli, denuded of chromatin and attached to the thin and barely visible nuclear envelope. No vacuole-like cavity is present in these nuclei. The cytoplasm is chromophobic in hematoxylin-eosin stained sections. In contrast to the dark type A spermatogonia, the cytoplasm of the pale type A cells is usually free from glycogen.

In Epon embedded sections examined by light microscopy the cell shape appears to be almost cuboidal (Fig. 17). The nucleus is spherical containing a homogeneous nucleoplasm. The nucleoli are peripherally located and are irregularly shaped. The cytoplasm is lightly stained and mitochondria appear to be dispersed throughout the cytoplasm. The nuclear:cytoplasmic ratio appears to be slightly less in these spermatogonia than in any of the other three types.

As shown by electron microscopy the cell shape of the Ap spermatogonium is somewhat spherical with a face adjacent to the basal lamina

(Fig. 25). The nucleus is also spherical and located in the center of the cell. The nucleus is homogeneous, without a region of rarefaction, and is lightly stained. Membrane specializations are not found on the nuclear envelope. The nucleoli are peripherally located within the nucleus and consist of an amorphous zone, generally associated with the internal face of the nuclear envelope, and a granular zone lying in the nucleoplasm (Fig. 6). In the region where the granular zone approaches the amorphous zone, a fibrillar region exists.

The overall density of the cytoplasm of the Ap spermatogonium is less than that found in the Ad type spermatogonium. The Ap mitochondria, with either tubular or plate-like cristae, are not associated with the nucleus but lie dispersed in the cytoplasm (Fig. 25). Subjectively, the quantity of mitochondria appears to be less in the Ap than in the Ad spermatogonium. The mitochondria of the Ap spermatogonium usually occur in pairs separated by an electron-opaque bar. Rarely are more than two mitochondria found together.

Paired centrioles have been observed in the now familiar right angle pattern (Fig. 25). The Golgi complex is similar in structure to that found in the Ad type spermatogonium. Single strands of rough and smooth endoplasmic reticulum are present in about equal amounts. Less glycogen is present in the Ap spermatogonium than in the Ad spermatogonium. Coated vesicles are routinely observed in the Ap cells.

The filamentous body, described above in the Ad spermatogonium, appears to be absent in the Ap spermatogonia although we have observed one filamentous body in a binucleate Ap spermatogonium. (Fig. 26):

The membranous body described in the AL spermatogonium does not appear to have a counterpart in the Ap spermatogonium. Finally, some myelin-like arrays, probably representing phospholipid degeneration, have been observed (similar structures are shown in the B type spermatogonia, e.g., Figure 28).

Type B (B) spermatogonia

In paraffin sections the type B spermatogonium displays a spherical nucleus containing some pale and some darkly stained chromatin granules (Fig. 27). Some of the deeply stained chromatin adheres to the nuclear membrane, which is clearly delineated. Its centrally located nucleolus is covered with granules. The cytoplasm is clear, and contains little or no glycogen. Although difficult to ascertain in paraffin sections the cell shape does appear to be pear-shaped.

Micron-thick Epon sections revealed the pear-shape nature of the B spermatogonia: In some instances the B spermatogonia do not appear to face on the basal lamina, however, by using serial sections it is clear that at least some portion of the B cell is in contact with the basal lamina; at times this connection is found to be only a "leg" of cytoplasm (Fig. 28). The nucleus is spherical and the nucleoplasm is homogeneous except for clumps of peripheral stained material. The nucleolus is always located in the central portion of the nucleus and is irregularly shaped. The cytoplasm is very lightly

stained and the mitochondria are dispersed throughout the cytoplasm.

Electron microscopic examination of the B type spermatogonium showed that the cell was pear-shaped and, when serial sections were examined, the cells were always found to have a connection with the basal lamina (Fig. 29). The nucleus shown in Figure 31 is somewhat tangentially sectioned and several pores in the nuclear envelope are shown. There is a slight amount of densely stained chromatin adhering to the internal face of the nuclear envelope which is characteristic of these cells. Nucleoli (Figs. 7 & 29) are centrally positioned and are composed of an amorphous zone that is surrounded by fibrillar and granular regions (Fig. 7). All of the nucleolus is quite electron dense. We have observed up to three separate nucleoli in the B nucleus.

The mitochondria are not joined to each other and their random location suggests that a preferential location of the mitochondria does not occur (Fig. 29). The mitochondrial cristae are primarily plate-like and there is no evidence of tubular cristae. There is an occasional association of the mitochondria with the rough endoplasmic reticulum. Both the smooth and rough variety of endoplasmic reticulum are present in only scanty amounts. Numerous unattached ribosomes are found in the cytoplasm of these cells. The Golgi complex is frequently more elaborate than found in the type A spermatogonia. Filamentous bodies are not present in the type B spermatogonia. There appears to be an increased number of myelin-like arrays in the B spermatogonia (Fig. 29) as compared to the type A spermatogonia. In

general, the cytoplasm is lightly stained and glycogen absent.

DISCUSSION

Four types of spermatogonia were found in normal men by a comparative study utilizing light and electron microscopy. Previously only three spermatogonial types were accounted for by definitive light microscopic studies. Light microscopy has not been sufficiently discerning to distinguish these four types of spermatogonia. It is eventually possible, however, that the many types described by Rosen-Runge and Barlow (1953) may actually be due to growth between mitoses.

The use of 1 μ thick Epon sections for light microscopy affords the visualization of considerably more cytological detail than can be obtained with paraffin embedded material and, consequently, four distinct types of spermatogonia can be identified in such Epon sections. However, the most satisfactory method for differentiating spermatogonial types was electron microscopic examination. This method showed an amazing amount of difference in the ultrastructural characteristic of the four types of human spermatogonia; almost every cell organelle appears to be modified to some extent within these spermatogonia. In addition, previously undescribed structures were found to occur only in AL and Ad spermatogonia. This study has relied essentially upon descriptive studies and the results in some cases seem to warrant speculation about function.

Relatively few cristae are present in inactive cells with low energy requirements (Fawcett, 1959 and 1966). The mitochondria of

all four spermatogonial types have few cristae. The sparse amount of endoplasmic reticulum of both the rough and the smooth variety would further suggest that these cells are inactive. The close apposition of the mitochondria to the rough endoplasmic reticulum, especially in the AL and Ad spermatogonia and less so in the Ap and B spermatogonia, would allow a short diffusion path between the mitochondria and the endoplasmic reticulum. Andre (1962) has suggested that a close association between mitochondria and endoplasmic reticulum might serve to supply energy for protein synthesis.

Whether the close apposition of the mitochondria to the nuclear membrane in the AL and Ad has any relation to the formation of the "stripe" or "bleb" found in the Ad is unknown. Whether this material is being excreted from the nucleus or absorbed into it is also speculative. Since the nucleolus at times is seen associated with the "stripes" (Fig. 21) an intriguing transport mechanism may be involved.

The function of the filamentous body, a characteristic structure in the AL and Ad spermatogonia, is unknown. The localization of ATPase in this body is suggestive evidence that it is an active center of energy utilization. The electron-opaque spheres found within this body are structurally similar in size and density to ribosomes.

The abundance of glycogen in the AL and Ad spermatogonia, and the lesser amounts of glycogen in the Ap and B spermatogonia, is taken as presumptive evidence that this substance is utilized as an energy source during spermatogonial development. Although occasional pino-

cytotic vesicles occur in these cells our evidence is not adequate enough to discount the movement of material in these vesicles in a reverse pinocytotic direction. One interesting facet about pinocytotic vesicles is that they occur only on adjacent cell-cell surfaces and not on the spermatogonial surface next to the basal lamina. This finding infers that transport occurs between the intercellular space and the spermatogonia.

An increased number of myelin-like arrays, thought to represent phospholipid degeneration, were present in the Ap and B spermatogonia, but not in the AL and Ad spermatogonia. This suggests that autolysis occurs in the more mature spermatogonia.

The Ad spermatogonia has been considered to be the most undifferentiated spermatogonial type (Clermont, 1963). Clermont (1966) suggests that the Ad spermatogonia have an "equivalent" mitosis and give rise to either two Ad spermatogonia or to two Ap spermatogonia. Each Ap spermatogonia subsequently gives rise to two type B spermatogonia which then give rise to the spermatocytes. This concept is that the Ad represents a cell that can either perpetuate the primitive stem cells or give rise to a spermatogonia, i.e., the Ap spermatogonia, that is committed to spermatogenesis. Our findings support and extend the portion of Clermont's work that suggests that there is a primitive spermatogonia, an intermediate form, and an advanced spermatogonia.

Knowing that spermatogonia located on the tubular wall eventually become the spermatids found in the lumen of the tubule it follows that the most advanced spermatogonia should show evidence of leaving the

walls of the seminiferous tubule. This is what is found in the B spermatogonium. This tear-shaped cell has a longitudinal axis perpendicular to the tubular wall and has little contact with the basal lamina. The amount of contact with the basal lamina decreases as the cells differentiate. The AL, Ad, Ap and B spermatogonia show decreasing amounts of contact with the basal lamina respectively.

Cytoplasmic and nuclear morphology also confirm that a progression of events occurs in spermatogonial development and is best described by considering the most undifferentiated spermatogonium to be the AL. For example, there is a progressive decrease in the number of mitochondria separated by electron-opaque bars from the AL to the Ad and Ap spermatogonia. The mitochondria of the B spermatogonia are not separated by electron-opaque bars. Perhaps also related to the above is the progressive decreased association of the mitochondria and the rough endoplasmic reticulum from the AL through the Ad and Ap to the B spermatogonia.

Another series of events ascribable to spermatogonial development is depicted by the nucleoli. In the AL spermatogonium the peripherally located nucleolus consists of a diffuse granular zone. In the Ad spermatogonium the granular zone is condensed but still maintains a peripheral position within the nucleus. A distinct amorphous region has not been observed within either the AL or the Ad spermatogonia. However, the peripherally located nucleoli of the Ap spermatogonium consists of a well-defined amorphous zone and condensed fibrillar and granular regions. In the B spermatogonium the nucleolus is also

composed of both an amorphous zone and fibrillar and granular zones, however, the position of the nucleolus is always central.

Thus, the cell shape and amount of contact with the basal lamina, the mitochondria-mitochondria relationship, the mitochondria-rough endoplasmic reticulum association, and the location and morphology of the nucleoli all suggest a sequence of spermatogonial development best explained by considering the AL spermatogonium to be the progenitor; that it gives rise to Ad spermatogonia which would subsequently give rise to Ap spermatogonia which would finally give rise to the most advanced spermatogonia, the type B.

We have found instances of binucleate AL, Ad, and Ap spermatogonia (e.g., see Figures 15, 24 & 25) and we interpret these cells as having completed karyokinesis and of partially complete cytokinesis. Fawcett (1969) has recently hypothesized that spermatogonia with mature nuclei are joined by intercellular bridges, a condition which exists throughout spermatogenesis. These binucleate cells would substantiate Clermont's mapping studies where pairs of spermatogonial types are found together and tend to confirm the occurrence of "equivalent" mitotic division in spermatogonia. We have never observed nuclei of different spermatogonial types within a common cytoplasm. Our present concept is that each cell mitotically divides to form two of the next more mature spermatogonia.

The correlation between our findings and Clermont's (1966) model of the mode of development and renewal of spermatogonia in man presents a tantalizing, and as yet unanswered, question. Is it possible that our AL spermatogonia represent primitive spermatogonia

that are capable of either regeneration of the stem cell population or of giving rise to the Ad spermatogonia which then form the Ap spermatogonia, etc. In this situation, the Ad spermatogonia would be the earliest spermatogonia committed to spermatogenesis. This possibility can be subjected to experimentation by depleting the spermatogonial population by irradiation and examining the tubule for remaining spermatogonia that must persist in order to give rise to spermatids by "recovery".

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- Figure 1. Light micrograph of AL type spermatogonium embedded in Epon. The flattened cell, the irregular shape of the nucleus, and the placement of the mitochondria in the cytoplasm away from the basement membrane lamina are distinguishing characteristics of the AL spermatogonia in micron-thick Epon sections.
- Figure 2. Electron micrograph of AL type spermatogonium. The spindle-shaped cell has been cross sectioned and the elliptical nucleus (N) appears spherical in this particular micrograph. The nuclear envelope is irregularly shaped and the nucleoplasm is homogeneous. The mitochondria (M) are linked by rough endoplasmic reticulum (R) and electron-opaque bars and show a preferential location away from the basal lamina (BM). The cytoplasm contains abundant glycogen (G) and a filamentous body (FB).
- Figure 3. Tangential sections of a typical nucleus (N) in AL type spermatogonium. A homogeneous nucleoplasm is shown within the irregularly shaped nuclear envelope. Abundant glycogen gives this cell a darker appearance. Golgi and mitochondria are present away from the basal lamina (BM).
- Figure 4. Nucleolus (Nu) of AL type spermatogonium. The nucleolus consists of a diffuse granular zone.
- Figure 5. Nucleolus (Nu) of Ad spermatogonium. The condensed granular zone is associated with the nuclear envelope.
- Figure 6. Nucleolus of an Ap type spermatogonium. The amorphous zone (A), in this micrograph, is associated with the nuclear envelope and a condensed network of the granular zone (GZ) lies in the nucleoplasm.
- Figure 7. Nucleolus of B type spermatogonium. The amorphous zone (A) is surrounded by a granular zone (GZ) and fibrillar region (F). The nucleolus is not associated with the nuclear envelope (NE).
- Figure 8. Mitochondria (M) of AL type spermatogonium. The mitochondria are frequently associated with the nuclear envelope (NE) and are joined together by rough endoplasmic reticulum (R). As shown by the circular profiles within the mitochondria (unlabeled) the cristae are tubular in nature. A Golgi complex (Go), a coated vesicle (CV) and a glycogen pocket (G) are shown.

- Figure 9. Electron-opaque bars (B) joining mitochondria (M) together in an AL type spermatogonium. A strand of rough endoplasmic reticulum (R) is associated with a mitochondrion.
- Figure 10. Electron-opaque bar (B) continuous with rough endoplasmic reticulum (R) in AL spermatogonium. Note the association of the bar with a mitochondrion (M).
- Figure 11. Longitudinal section of a filamentous body (FB) in the cytoplasm of an AL type spermatogonium. Electron-opaque spheres (Sp) are shown between the parallel filaments.
- Figure 12. Cross section of a filamentous body (FB) in the cytoplasm of an AL type spermatogonium. Electron-opaque spheres (Sp) can be observed within the encircling linear array of tubules.
- Figure 13. Longitudinal section of a filamentous body (FB), in the cytoplasm of an AL spermatogonium. The tissue was incubated for ATPase activity and reaction product is shown to be localized within this filamentous body.
- Figure 14. Lamellar body (L) in the cytoplasm of an AL spermatogonium. The lamellar body is limited by a single membrane and shows an internal lamellar system composed of membranes. Mitochondria (M) and elaborate Golgi (Go) are also present.
- Figure 15. Two AL nuclei (N_1 and N_2) in a common cytoplasm. Both the cytoplasm and the nuclei possess the characteristics of AL spermatogonia. The cell lies on the basal lamina (BM).
- Figure 16. Light micrograph of an Ad type spermatogonium and an Ap type spermatogonium embedded in paraffin. Note the "vacuole" in the nucleus of the Ad.
- Figure 17. Light micrograph of an Ad type spermatogonium and an Ap type spermatogonium embedded in Epon. Note the rarefied (R) area in the Ad nucleus and the length of these cells on the basal lamina (BM).
- Figure 18. Electron micrograph of an Ad type spermatogonium. Contact of the spermatogonium with the basal lamina (BM) is evident. The nucleus (N) contains granular nucleoplasm, a region of rarefaction (RR), and a peripheral nucleolus. Mitochondria (M) are shown in close proximity to the nuclear envelope and are also joined to each other. Smooth endoplasmic reticulum (S) is shown in the region of the centriole (C) and the Golgi complex (Go).

- Figure 19. Nucleus in Ad spermatogonium. A peripheral nucleolus is shown as well as a portion of a nucleolus (Nu) within the region of rarefaction (RR). A membranous elaboration (E) is associated with the nuclear envelope.
- Figure 20. "Stripe" associated with the nuclear envelope of an Ad type spermatogonium. The nuclear envelope is continuous and the stripe is composed of a membrane limited cisternum (C) and dense layers (DL) on the internal side of both the nuclear envelope and the cisternum. The nucleoplasm, (N) and cytoplasm (CY) are indicated.
- Figure 21. Nucleolus (Nu) associated with stripe (St) on the nuclear envelope of Ad spermatogonium.
- Figure 22. "Bleb" associated with the nuclear envelope (Ne) of an Ad type spermatogonium. The nuclear envelope is continuous and protrudes into the cytoplasm (CY). The membrane specialization (bleb) is composed of a double membrane limited body having particulate and filamentous contents. Note that a dense layer (DL) separates the nuclear envelope from the bleb.
- Figure 23. "Blobs" and "stripes" associated with the nuclear envelope of an Ad type spermatogonium. This micrograph suggests that blebs (BL) and stripes (St) may be related, but represent different stages of development.
- Figure 24. Two Ad nuclei (N_1 and N_2) linked by a cytoplasmic bridge (CB). The nuclei are identified primarily by the region of rarefaction (RR) that is found only in the nuclei of this spermatogonial type.
- Figure 25. Electron micrograph of an Ap type spermatogonium. The cell is in contact with the basal lamina (BM). The nucleus is spherical, the nucleoplasm homogeneous, and the multiple nucleoli are peripheral. The cytoplasm contains little glycogen and is somewhat lighter in density than the cytoplasm of either of the other A spermatogonia. A pair of centrioles (C) is present and the mitochondria (M) are frequently joined in pairs. The nucleus (NS) of a Sertoli cell is indicated.
- Figure 26. Binucleate Ap type spermatogonium. The nucleoli are peripherally located and a pars amorpha (P) and condensed nucleolonema (NL) are present. A filamentous body (FB), a structure not generally found in Ap type spermatogonia, is present.

Figure 27. Light micrograph of B type spermatogonium embedded in paraffin.

Figure 28. Light micrograph of B type spermatogonium embedded in Epon.

Figure 29. Electron micrograph of B type spermatogonium. The pear-shaped cell has a minimal, yet definite, contact with the basal lamina (BM). The nucleolus is centrally positioned and flakes of chromatin (Ch) are shown on the internal face of the nuclear envelope. The mitochondria (M) are not joined together and occur throughout the cytoplasm. Scanty amounts of both smooth (S) and rough (R) endoplasmic reticulum and a Golgi complex (Go) are present.

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APPENDIX C

PREPRINT OF PAPER SUBMITTED TO LABORATORY INVESTIGATION

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COMPARATIVE MORPHOLOGICAL AND FUNCTIONAL STUDIES OF
NEPTUNIUM-INDUCED FATTY LIVERS IN RATS*

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ABSTRACT

The effect of neptunium-237 on rat liver was studied using electron microscopic techniques. Crescent shaped dilatations of the endoplasmic reticulum were the earliest morphologic alterations observed and were found two hours after ²³⁷Np administration. By 24 hours post-injection, large quantities of lipid was present in the liver and two types of lipid bodies were observed under the electron microscope. The one type was electron-transparent and associated only with the endoplasmic reticulum; the other was electron-opaque and associated with both the endoplasmic reticulum and the Golgi complex.

Glucose-6-phosphatase, found within the cisternae of the endoplasmic reticulum in control liver was displaced in animals treated with ²³⁷Np, but treatment of the rats with butylated hydroxytoluene prior to ²³⁷Np injection prevented the displacement of glucose-6-phosphatase.

Key words: Fatty liver, Electron microscopy, Antioxidant, Glucose-6-phosphatase.

*This paper is based on work performed under United States Atomic Energy Commission Contract AT(45-1)-1830.

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COMPARATIVE MORPHOLOGICAL AND FUNCTIONAL STUDIES OF
NEPTUNIUM-INDUCED FATTY LIVERS IN RATS*

by

J. D. Berlin and D. D. Mahlum

Many toxic materials, including carbon tetrachloride (CCl_4), ethionine, crotonic acid and puromycin, induce fatty livers in rats with associated alterations in subcellular organelles.^{6,15} In particular, dilation of the rough endoplasmic reticulum and dissociation of ribosomes from the membranes have been reported;^{1,2,10,32} a decreased synthesis of proteins, particularly the plasma lipoproteins accompanies these morphological alterations.^{27,31,35,37} These observations among others led Lombardi and Recknagel¹⁶ to postulate that impaired secretion of lipid from the liver was a common feature in the pathogenesis of fatty livers produced by many hepatotoxins.

Recent attention has focused on the possible causal role of lipoperoxidation in the morphologic and biochemical disturbances observed after the administration of CCl_4 , ethanol and crotonic acid.^{5,14,22} Indirect evidence for the possible role of lipoperoxidation has been available for a number of years from experiments in which the administration of antioxidants such as a tocopherol and DPPD ameliorated the hepatotoxic effects of CCl_4 at both the histologic and biochemical levels.^{7,9} More recently, Recknagel and Ghoshal^{23,24} showed that CCl_4 acted in vitro and in vivo as a prooxidant for microsomal lipids. Increased levels of lipoperoxides have been found in livers of rats treated with ethanol or crotonic acid.^{4,12,14}

*This paper is based on work performed under United States Atomic Energy Commission Contract AT(45-1) 1820

Previous work in this laboratory¹⁹ demonstrated that the administration of the actinide element ^{237}Np to rats produced severe hepatic damage. This effect was more pronounced in the female than in the male and was characterized by neutral fat accumulation. It was also demonstrated that the effect of ^{237}Np could be ameliorated by prior treatment of the animals with butylated hydroxytoluene (BHT).¹⁸ These morphologic effects of ^{237}Np were previously studied histologically. The present investigation was, therefore, initiated to determine if the ultra-structure of the hepatic cell also underwent changes after ^{237}Np administration which were similar to those produced by other hepatotoxins.

Alterations in the rough endoplasmic reticulum (RER) were observed in the liver from ^{237}Np -treated rats during the early phases of this investigation; therefore, a study was made of the localization of glucose-6-phosphatase which was considered as a marker enzyme for the RER.³² Carbon tetrachloride has been reported to cause decreases in the levels of this enzyme²⁵ but effects on the subcellular localization have not been reported. The effect of BHT on the localization of G-6-Pase was examined in an attempt to explain the ameliorating effect of this antioxidant on the toxic action of ^{237}Np . In addition, the incorporation of ^{14}C -leucine into hepatic and plasma proteins was studied to determine if the morphologic alterations in the RER were accompanied by changes in protein synthesis.

MATERIALS AND METHODS

Female rats weighing 250-350 grams were injected intravenously with either 12 or 24 mg/kg of body weight of ^{237}Np as the citrate; these rats were sacrificed at 3 or 24 hours after ^{237}Np injection. In the studies in which BHT was used, rats were treated with 20 mg/kg 19 hours and 0 and 8 hours subsequent to ^{237}Np injection and sacrificed 24 hours after

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²³⁷Np administration. Control animals were given a comparable citrate solution without ²³⁷Np. Since rats ordinarily do not eat during the first 24 hours following injection with ²³⁷Np, all animals were fasted after injection.

Electron Microscopy:

The tissues to be studied morphologically were fixed in 1% glutaraldehyde on 0.1 M cacodylate buffer (pH 7.3) for 1 hour at 4°C, postfixed in osmium tetroxide ²⁰ for 1 hour at 4°C, dehydrated in ethanol and embedded in Epon.¹⁷ For the cytochemical studies, the tissues were initially fixed in 1% glutaraldehyde as above, and rinsed in 0.1 M cacodylate buffer 5% sucrose at pH 7.3 for 2 1/2 hours prior to incubation in medium containing glucose-6-phosphate ³⁵ at 37°C for 13 minutes. After incubation, the tissues were rinsed twice in the sucrose containing buffer and post-fixed in osmium tetroxide,²⁰ dehydrated and embedded as above. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate²⁵ and examined in an RCA EMU-2C or -3B electron microscope.

Protein Synthesis:

The incorporation of ¹⁴C-labeled leucine into liver and plasma proteins was used as a measure of protein synthesis. Female rats were injected with labeled amino acid either 5 or 24 hours after ²³⁷Np (12 mg/kg body weight) administration; plasma and liver samples were obtained one hour later. The liver samples were homogenized in water and the proteins precipitated by the addition of an equal volume of 10% TCA; TCA was added directly to the plasma samples. The precipitates were washed once each with TCA, ethanol: ether (3:1), and ether; the protein was then dissolved in 0.5 N NaOH. Protein determinations were performed by the biuret method.⁸

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The presence of ^{237}Np in the liver and plasma samples necessitated the conversion of the protein samples to CO_2 before counting to eliminate the contribution from ^{237}Np . This was accomplished by modification of the method of Kelly¹³ in which the samples were placed on black filter paper, dried and combusted in an atmosphere of oxygen. The CO_2 produced was absorbed into a mixture of ethanolamine and ethanol (1:2) and an aliquot counted in a liquid scintillation counter using a scintillator solution of 3 grams of terphenyl and 0.03 grams POPOP dissolved one liter in toluene. This procedure proved extremely effective, reducing contamination from ^{237}Np to less than 0.1%.

RESULTS

The subcellular morphology of the control rat hepatocytes was essentially identical to that reported for liver cells in the literature. Therefore, a general description of the hepatocyte will be omitted and the reader is referred to reviews by Novikoff and Essner²¹ and Rouiller and Jezequel³⁰ for this information.

Structural Alterations Induced by ^{237}Np :

The earliest changes in hepatocyte structure of ^{237}Np -treated rats were dilatations of the rough endoplasmic reticulum (Fig. 1) observed at 3 hours after treatment. These dilatations which are termed "Crescents" because of their characteristic profiles, were routinely present at 3 hours but rarely seen at 24 hours after ^{237}Np injection. They were approximately 0.2 μ wide and 1 μ long and their interiors always appeared electron-transparent by the methods of preservation used in this study. The crescents were discreetly localized in a few cisternae of the rough endoplasmic reticulum and the general parallel orientation of cisternal stacks was maintained (Fig. 1). The ribosome population of the membranes

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limiting the crescents compared to typical rough endoplasmic reticulum, it was not determined whether this was a true loss of ribosomes or only an apparent decrease in ribosome numbers resulting from an increased surface area of the membrane. Rough endoplasmic reticulum cisternae continuous with and adjacent to the crescents were morphologically unaltered that the usual complement of ribosomes (Fig. 1). Crescents similar to those in the rough endoplasmic reticulum were also observed within the nuclear envelope. Hepatocytes from 2 and 3 hours ²³⁷Np-treated rats did not contain increased numbers of typical lipid inclusions, although the crescents may represent an initial lipid accumulation. With the exception of the above described crescents the architecture of the hepatocyte appeared normal 3 hours after treatment and no changes were observed in other cellular structures.

In rats sacrificed at 24 hours after ²³⁷Np, two types of bodies, both presumably lipid in nature, were observed in the hepatocytes. The two types of bodies were never found within the same cell. The first type was a sphere of approximately 1-4 μ diameter and appeared electron-transparent and membrane-limited (Fig. 2 and 3). These bodies were frequently joined in an apparent anastomotic condition and sometimes observed to be continuous with the crescents in the rough endoplasmic reticulum. Hence, the limiting membrane of the electron-transparent bodies appeared to be continuous with the membrane of the rough endoplasmic reticulum via the membrane lining the crescents.

The second type of body was electron-opaque, 0.1-0.5 μ in diameter, (Fig. 4 and 5), and usually found within the rough endoplasmic reticulum although they also occurred individually and in clusters throughout the cytoplasm. An occasional association of these electron-opaque bodies with the Golgi complex was observed (Fig. 5). Submersion of sections

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in xylene for 1 hour to extract lipid caused the electron-opaque structures to become electron-transparent (Fig. 6).

At 23 hours after ^{237}Np injection, the rough endoplasmic reticulum in the hepatocyte was disoriented and the integrity of the parallel arrays was rarely maintained. Quite frequently cisternae of the rough endoplasmic reticulum were closely associated with mitochondria (Fig. 4). In spite of the apparent disorganization of the cisternae, ribosomes remained associated with the rough endoplasmic reticulum at all times examined. Definite structural alterations in other cellular structures were not observed at this time period.

Localization of Glucose-6-Phosphatase:

In control rats, glucose-6-phosphatase activity was localized within the cisternae of the rough endoplasmic reticulum (Fig. 7 242-x3). The deposition of the reaction product in small patches along the length of the endoplasmic reticulum is a reflection of the short incubation time used. Occasionally it appears to be localized on the internal face of the membranes, suggesting that the glucose-6-phosphatase is attached to the membranes. Regardless of the precise location of the enzyme within the endoplasmic reticulum in control animals, it is separated from the ground substance by a membrane and the reaction product and was not found free in the cytoplasm or associated with any cell organelle other than the rough endoplasmic reticulum.

After treatment with EHT alone, glucose-6-phosphatase localization was similar to that observed in the control animal (Fig. 8). The reaction product was found in the rough endoplasmic reticulum and appeared to be associated with the internal face of the limiting membrane. Similarly, the fine structure of the hepatocytes resembled that found in control rats

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and no discernible subcellular alteration, either morphological or cytochemical, was observed as a result of EHT treatment.

In hepatocytes from rats treated with ^{237}Np for 24 hours, the reaction product for glucose-6-phosphatase was rarely observed within the cisternae of the endoplasmic reticulum (Fig. 9 and 10 2703A). Occasionally the precipitate appeared to be loosely associated with the external face of the membrane of the endoplasmic reticulum, but more routinely appeared to lie free in the ground substance. Although displaced from its original position within the rough endoplasmic reticulum, the enzyme was not found associated with any other cell organelle.

This dislocation of the enzyme by ^{237}Np treatment was prevented by treatment of the animals with EHT. Thus, in rats treated with EHT 19 hours prior to, as well as at 0 and 8 hours after ^{237}Np administration, and sacrificed 24 hours after ^{237}Np treatment, the reaction product for glucose-6-phosphatase was found within the cisternae of the rough endoplasmic reticulum, (Fig. 11), and was never observed within the ground substance.

^{14}C -Leucine Studies:

Although rather obvious morphologic changes had occurred in the liver by 3 hours after ^{237}Np injection, little difference was found between the incorporation of ^{14}C -leucine into liver and plasma proteins of control animals and those pretreated for 5 hours with 12 mg/kg ^{237}Np (Table 1). At 24-hour pretreatment with ^{237}Np resulted in a 50% decrease in the incorporation into liver proteins but did not reduce the activity of the plasma proteins.

DISCUSSION

The structural alterations found in neptunium-induced fatty livers

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are in general accord with the morphological changes reported to occur with other fatty liver agents (CCl_4 , ethionine, orotic acid).^{1, 2, 10, 32} The first morphological indication of hepatic injury is a disruption of the endoplasmic reticulum including the loss of ribosomes from the membranes, dilated cisternae, and a loss in the integrity of the cisternal stacks. At a later time, lipid bodies are found in increased numbers. The crescents that occur in ^{237}Np -induced fatty livers represent a slight morphological variation from that observed in fatty livers produced by other agents. Dilations of the endoplasmic reticulum that have been described in other types of fatty livers lack the very definite structure displayed by the crescents. The functional significance of the crescents is not known although the direct continuity between the crescents and the electron-transparent lipid bodies suggests that the crescents may represent an initial manifestation of lipid accumulation in the liver.

We suggest that both the electron-opaque and the electron-transparent bodies that were observed in the 24 hour ^{237}Np -treated rats are lipoidal in nature with their different electron-densities reflecting different chemical characteristics. The two types of bodies were never found within the same cell suggesting that different areas of the liver lobule may be engaged in the synthesis of different types of lipid. Unfortunately, the present study did not differentiate between the peripheral and central regions of the liver.

Electron microscopists have noted for a number of years the presence of electron-dense granules of 800-1000 Å diameter associated with the Golgi apparatus endoplasmic reticulum and the space of Disse^{3, 34} in rat liver cells. Jones et al.,¹¹ found an increased number of these granules after perfusion of the liver with a lipid-rich medium and concluded that they

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were lipoproteins. The electron-opaque bodies in the livers of rats treated with ^{237}Np in the present study were also associated with both the endoplasmic reticulum and Golgi complex, but were not found in the space of Disse. These bodies may consist of lipoproteins which accumulate in the hepatocyte due to a failure of the secretory mechanism. This speculation, if proven, would help to explain the mechanism of fatty liver development after ^{237}Np administration. The present data is insufficient to confirm this hypothesis.

The nature of the electron-transparent bodies is less clear. It is possible that the contents of these bodies, as well as the contents of the crescents, have been extracted by our method of sample preparation. The collapse of the crescent membrane, which was a characteristic finding, lends support to this hypothesis. If the contents have not been leached, the electron-transparent bodies would be presumed to contain fewer unsaturated lipids than the electron-opaque bodies by virtue of their lack of osmium uptake.

In any case, the formation of the electron-transparent bodies seems to be directly associated with the endoplasmic reticulum and does not appear to require the Golgi complex.

An inhibition of protein synthesis has been causally associated with the fatty livers observed after administration of CCl_4 ,^{31,32} ethionine,³⁵ puromycin²⁷ and orotic acid.³⁷ This inhibition of protein synthesis has been shown, especially in the case of CCl_4 , to be related to loss of ribosomes from the endoplasmic reticulum. In the present study, denudation of the endoplasmic reticulum was less than reported by Smuckler et al.³² after CCl_4 administration to rats. Inhibition of ^{14}C -leucine incorporation into either liver or plasma proteins was also less than reported for other hepatotoxins which would correlate with the morphologic

findings. Although the incorporation of leucine into plasma proteins was not decreased even at 24 hours after ^{237}Np administration, it should be noted that individual fractions such as the low density lipoproteins were not analyzed. It has been shown by Foheim et al. ^{28, 29} that incorporation of a labeled amino acid into plasma lipoproteins may be inhibited without overall inhibition of protein synthesis.

Since the endoplasmic reticulum appeared to retain at least some of its morphological and functional characteristics as indicated by the presence of ribosomes and the amino acid incorporation data, we examined another measure of the integrity of this organelle. The localization of glucose-6-phosphatase activity was selected because this enzyme is associated with the endoplasmic reticulum and because it can be determined with relative ease by electron microscopic cytochemistry. The confinement of G-6-Pase within the cisternae of the endoplasmic reticulum in control hepatocytes is in agreement with the concept that this enzyme is a marker enzyme of this organelle. The displacement of G-6-Pase from within the cisternae of the endoplasmic reticulum by the administration of ^{237}Np suggests that major alterations have occurred in this structure. Although it has been reported previously that CCl_4 treatment decreases the total G-6-Pase activity, ²⁵ there has not been to our knowledge a report of a change in the intracellular localization of the enzyme due to the administration of a hepatotoxin.

Previous work in this laboratory showed that BHT inhibited liver lipid accumulation after ^{237}Np administration, although it did not prevent the rise in levels of isocitric dehydrogenase or glutamopyruvate transaminase levels found in the serum of ^{237}Np -treated rats. ¹⁸ In the present study, BHT prevented the displacement of G-6-Pase from the cisternae of the ER.

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FIGURE LEGENDS

Figure 1. Crescent (C) in cisternum of rough endoplasmic reticulum of hepatocyte from rat sacrificed 3 hours after ^{237}Np treatment. The collapse of the membrane limiting the crescent (arrows) is thought to be the result of dissolution of the crescent contents during sample preparation. Ribosomes are present on the membrane limiting the crescent, however, the number of ribosomes on these membranes appears to be reduced as compared to "typical" rough endoplasmic reticulum. The rough endoplasmic reticulum that is continuous with the crescent, as well as the adjacent cisternal elements, appear morphologically unaltered and possess the usual complement of ribosomes.

Figure 2. Electron-transparent lipid bodies (L) in hepatocyte of 24 hour ^{237}Np -treated rat. Several lipid bodies are shown to be continuous with each other and with crescents (C) in the rough endoplasmic reticulum.

Figure 3. The morphological relationship between the electron-transparent lipid bodies (L) and the crescents (C) in hepatocyte of 24 hour ^{237}Np -treated rat is shown. Both the contents and the membranes of these structures are continuous.

Figure 4. Electron-opaque lipid bodies in hepatocyte of 24 hour ^{237}Np -treated rat are shown within the rough endoplasmic reticulum (arrows) which is frequently associated with mitochondria (M). Similar electron-opaque lipid bodies (L) also occur individually and in clusters throughout the cytoplasm.

Figure 5. Clusters of electron-opaque lipid bodies (L) are occasionally observed to be continuous with cisternae of the Golgi complex (G) in 24 hour ^{237}Np -treated rats.

Figure 6. This electron micrograph demonstrated the lipoidal nature of the electron-opaque bodies in hepatocyte of 24 hour ^{237}Np -treated rat. The section was submerged in xylene for 1 hour and the electron-opaque structures became electron-transparent. Compare with Figures 4 and 5.

Figure 7. Glucose-6-phosphatase localization in hepatocyte from control rat. The reaction product occurs in the intracisternal portion of the rough endoplasmic reticulum and is not associated with any other cellular organelle.

Figure 8. Glucose-6-phosphatase localization in hepatocyte from rat treated only with the antioxidant BHT. The reaction product is confined to the rough endoplasmic reticulum.

Figure 9. Survey electron micrograph of glucose-6-phosphatase localization in hepatocyte from 24 hour ^{237}Np -treated rat. The reaction product is not found within the rough endoplasmic reticulum nor within any other cell organelle including mitochondria (M), microbodies (Mb), the nucleus (N), Golgi complex (G) and lipid bodies (L).

Figure 10. Glucose-6-phosphatase localization in hepatocyte from 24 hour ^{237}Np -treated rat. The reaction product rarely occurs within the cisternae of the rough endoplasmic reticulum, however, it can be observed in the ground substance.

Figure 11. Glucose-6-phosphatase localization in hepatocyte of rat treated with BHT (43 hours) and ^{237}Np (24 hours) prior to sacrifice. The reaction product occurs within the cisternae of the rough endoplasmic reticulum and not in the ground substance.

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Table 1. Effect of ^{237}Np of Incorporation of ^{14}C -leucine into Proteins of Liver and Plasma

^{237}Np	Time after Administration hrs.	Liver		Plasma	
		Control	^{237}Np c.p.m./mg protein	Control	^{237}Np
	5 ^a	147	156	410	505
	24 ^b	98	52	133	159
	24 ^c	31	24	78	86

^a Each rat received 10 μCi of DI- ^{14}C -leucine intramuscularly (3 controls and 3 treated).

^b Each rat received 5 μCi of L- ^{14}C -leucine intravenously (6 controls and 6 treated).

^c Each rat received 2 μCi of L- ^{14}C -leucine intravenously (6 controls and 6 treated).