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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

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Authentication:

Jerry D. Berlin
Principal Investigator

Monty Davenport
Dr. Monty Davenport
Associate Vice President
for Research

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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

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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.

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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; Leeson 1966; Nagano, 1962, 1966; de Kretser, 1967a, 1967b; Smith Leeson, and Bunge, 1967; Smith, Leeson, Bunge, and Anderson, 1967; Yasuzumi, Nakai, Tsubo, Yasuda, and Sugioka, 1967; Tres and Solari, 1968; Berlin, 1968a,

and Berlin and Adee, 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 Barrnett, 1963; Gordon and Barrnett, 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

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% $CaCl_2$ (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

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 brillant 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 brillant 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.

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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.

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

	Estimated Requirements 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)		$ 2450.00
(1/5 time for 9 months)	$ 2939.00	
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

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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)

	Estimated Requirements 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)		$ 2573.00
(1/5 time for 9 months)	$ 3087.00	
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

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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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