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EFFECTS OF IONIZING RADIATION ON THE TESTICULAR FUNCTION OF MAN

AT (45-1) 1780

9 YEAR PROGRESS REPORT-May 1972

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I. OBJECTIVES

A. ORIGINAL OBJECTIVES

In the initial grant request we proposed to apply known amounts of ionizing radiation directly to the testes of normal men in order to ascertain specific cytological and hormonal information. With respect to the cytological information, we proposed: (1) to determine the exact nature of the cytological defect produced in the development of the germinal epithelium and to relate the extent of the defect to dosage and time; (2) to find the minimal dosage (and thereby determine dosage tolerance) that will affect the germinal epithelium; (3) to determine the time of recovery from any given dosage; (4) to determine the minimal dosage that leads to permanent damage of spermatogenic cells; (5) to determine the simultaneous effects of any dosage upon Leydig cell cytology.

With respect to hormonal information, we proposed to determine the influence of any given radiation-produced testicular alteration upon other parameters such as (6) total gonadotropin and (7) interstitial cell-stimulating hormone (ICSH) excretion, (8) estrogen excretion, and (9) androgenic hormone excretion.

B. ADDITIONAL OBJECTIVES AND CHANGES IN TECHNIQUES

As the work progressed and as suggestions were made by the Advisory Committee of the AEC (meeting in Seattle, November, 1963, December, 1965, March, 1967 and December, 1967), some parameters were added and some dropped.

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Subsequently it was proposed to emphasize the delineation of the cytological defect: by quantitating the number of recognizably damaged cells during the first 16 hours following radiation; by determining the number of remaining cells of each cell type during the denuding period (the first 46 days); and by assessing the problem of spermatogonial renewal in man.

Cytogenetics was introduced as a new parameter in order to harvest the greatest amount of information from the continuing investigation. The purpose of the cytogenetics was to analyze chromosomal abnormalities following irradiation during meiosis and during mitosis, if possible.

Early in the investigation we observed a rise in total urinary gonadotropins following any radiation dose causing denuding of the germinal epithelium. Concurrently no change in urinary ICSH was observed. In order to confirm which gonadotropin was involved we began measuring urinary FSH separately using the Steelman-Pohley assay method (1). The objective was to affirm whether the germinal epithelium utilized FSH, since urinary ICSH did not change and urinary total gonadotropins increased. We have begun measuring plasma FSH by radioimmunoassay. This method has enabled us to confirm and add to our data obtained from urinary observations of FSH. Since observations on the reduction of urinary testosterone suggested that Leydig cells were affected by radiation, additional parameters were added to measure this effect. These included quantitation of Leydig

cells, radioimmunoassay of plasma ICSH and measuring plasma testosterone by a competitive protein binding method. Currently an ultrastructural study of Leydig cells is underway to determine any ultrastructural changes in morphology of these cells following irradiation.

C. ABANDONED OBJECTIVES

For two years we conducted exploratory investigations on the mapping of meiotic (pachytene) chromosomes in order to establish a basis for the evaluation of irradiation effects. Neither sufficient precision nor quantitative data resulted. Observing the usual parameters or chromosomal breaks, bridging, translocation and other gross chromosomal defects confirmed that, in man, as in other species, radiation at each dose level (including 10r) caused damage. Since nothing new was being revealed, and following discussion with the AEC Advisory Committee team (Seattle, March, 1967), this approach was abandoned.

The same committee, being intrigued with the finding of lowering of urinary testosterone values following irradiation, suggested that metabolic defects in the testicular production of testosterone may be involved. It was proposed that studying urinary pregnanediol and pregnanetriol might reveal the metabolic defect as was found in rats by Berliner, et al. (2) . Hence these two parameters were measured. Finding no change, this pursuit has also been abandoned.

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II. MAIN RESEARCH ACCOMPLISHMENTS

A. STATUS OF X-RAY IRRADIATED SUBJECTS

1. Subjects Irradiated

Total number of subjects irradiated ---------- 74

8r	4	78r	7
10r	3	100r	10
15r	1	200r	13
20r	8	235r	1
25r	2	300r	2
50r	5	400r	2
5r/11 weeks = 55r	1	600r	15

Seven subjects were irradiated again following complete recovery from the initial dosage. Of these, three received the identical dosage on each of two occasions, three received two different dosages, and one received three different dosages. Each dosage is listed separately above. All proposed irradiations have been completed.

2. Biopsy Data

Number of subjects in whom serial biopsies were taken throughout the irradiation period ---------- 42

Number of subjects in whom biopsies were avoided following irradiation in order to specifically evaluate sperm and hormonal alterations ---------- 13

Number of subjects in whom biopsies were avoided only during the first 90 day cell depletion period following irradiation ------ 19

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3. Vasectomy Data

Number of subjects having had pre-irradiation vasectomies ---------- 12

Number of subjects vasectomized before release ---------- 42

Number of subjects not vasectomized before release ---------- 0

4. Status of Program

Number of subjects who completed recovery from irradiation before release ---------- 22

Number of subjects released before complete recovery from irradiation ---------- 26

Number of subjects who returned after release but before recovery was completed and the investigation continued -------- 5

Number of subjects currently under investigation ---------- 21

B. GENERAL STATEMENT

For each parameter studied we have found that each subject must serve as his own control. This has been confirmed as results have been analyzed statistically. Moreover it has been established, upon statistical bases, that the number of control observations necessary for each parameter will depend largely upon the nature of the parameter. For example, a single control testicular biopsy usually suffices, whereas a minimum of 18 control serial weekly seminal fluid examinations are necessary to establish a base line.

For each method of analysis and for each approach much time and effort has therefore been devoted to delineating the limits and

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variations of the normal physiology of reproduction. As a result, new methods of approach have been worked out and new physiological principles have been uncovered or more precisely defined.

As a consequence of this attention to the normal, many of our initial publications deal with such problems. Examples are: "The testicular biopsy: surgical procedure, fixation and staining technics", Rowley, M. J. and Heller, C. G., Fertil. Steril., 17:177, 1966 (3). "Decreases in sperm concentration due to testicular biopsy procedure in man", Rowley, M. J., O'Keefe, K. B. and Heller, C. G., J. Urol., 101:347, 1969 (4). "Human spermatogenesis: An estimate of the duration of each cell association and of each cell type". Heller, C. G., Heller, G. V. and Rowley, M. J., Progress in Endocrinology, III International Congress of Endocrinology, Excerpta Medica International Series, 184, 1012 (5). "Duration of transit of spermatozoa through the human male ductular system", Rowley, M. J., Teshima, F. and Heller, C. G., Fertil. Steril., 21:390, 1970 (6). "A method for the quantification of Leydig cells in man", Heller, C. G., Lalli, M. F., Pearson, J. E. and Leach, D. R., J. Reprod. Fert., 25:177, 1971 (7). "The ultrastructure of four types of human spermatogonia", Rowley, M. J. and Heller, C. G., Z. Zellforsch., 112:139, 1971 (8). "Quantitation of the cells of the seminiferous epithelium of the human testis employing the Sertoli cell as a constant". Rowley, M. J. and Heller, C. G., Z. Zellforsch., 115:461, 1971 (9).

Another developmental aspect, regarding the radiation program, was to solve the problem of delivering as uniform an amount of radiation as possible to all depths of testicular tissue of both testes without

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exposing the subject to any extraneous radiation. We rejected the conventional X-ray therapy units on the market as unsuitable because of lack of means of uniform coverage of the testes, lack of assurance that uniformity from subject to subject could be established and lack of (or awkward) shielding protection to the subject's body. A simple portable box was designed by Peter Wootton (physicist) that allowed the scrotum and testes to drop into a plastic box of water at scrotal temperature and then be irradiated by two X-ray tubes. The tubes delivered measured amounts of known quantities of irradiation to the two submerged testes simultaneously from two directions without (or with the most minimal) exposure to the pelvic region. This has been summarized as "The effects of graded doses of ionizing radiation on the testicular function of man. I. A portable device for delivery of uniform doses of X-ray irradiation throughout externalized organs", Wootton, P. and Heller, C. G., and is unpublished to date (10).

The accuracy of delivery of this X-ray irradiation device has been confirmed by physical as well as biological dosimetry. The physical check was seen by a team from the Hanford Washington AEC group under the supervision of Dr. William Roesch. The biological check was run by Dr. Eugene Oakberg, Oakridge, using well standardized male mice. These were submerged (in specially designed containers) into the plastic box in the same position as the scrotal testes. They were exposed to a similar series of graded doses of radiation as the subjects' testes. The results were comparable to similar studies at Oakridge, Berkeley, and M.I.T., and verified the accuracy of exposure.

See Fourth Yearly Progress Report for details (1966-67).

Our past data has been statistically evaluated with the view to adding observations to each parameter, where required, in order to assure a statistically acceptable result. For example, in order to consolidate information regarding hormonal changes resulting from X-ray exposure to the testes, a low (8r), a medium (78r) and a high dose (600r) were selected. It was decided that urine collections for hormonal analyses must be made in eight day pools repeated six times during the control period in order to establish a statistically valid base line for each subject for comparison with post-irradiation results. The number of subjects per dose and the number of post-irradiation observations were also pre-determined. Some of the studies have been completed, others are currently being performed and others are projected. In the end, we expect to have a statistically significant insight into the hormonal changes. With the same goal in mind we have similarly applied statistical analysis to our germinal and Leydig cell quantitation and sperm count evaluations.

C. ACCOMPLISHMENTS WITH SPECIAL REFERENCE TO ORIGINALLY STATED OBJECTIVES AND PLANS FOR CONTINUATION OF PRESENT OBJECTIVES.

1. Cytological Objectives

a. Objective: "To determine the exact nature of the cytological defect produced in the development of the germinal epithelium and to relate the extent of the defect to dosage and time."

In the following discussion dosage has been divided into "low", "intermediate", and "high", according to the cytological response elicited by exposure to each dosage.

Low dosage effects (10 - 100r): Examination of serial testicular biopsies following exposure to irradiation, revealed that the spermatogonia were primarily affected, and that more mature cells were allowed to complete normal development. The overt damage to the spermatogonia was revealed by pyknosis and other signs of degeneration. The concealed damage to otherwise normally appearing spermatogonia became overt as revealed by the failure of the cells to undergo mitosis and produce preleptotene spermatocytes. At the same time (for dosages of 100r or less) the preleptotene, other spermatocytes and the spermatids failed to reveal either overt or concealed damage. The latter was deduced from their ability to undergo development and maturation (including undergoing maturation-division) to become normal mature spermatozoa and to appear in the ejaculate as normal spermatozoa in regard to numbers as well as morphology. Since the preleptotene spermatocytes were not replaced by the spermatogonia, however, the production of sperm ceased when depletion was completed. Thus the three consequences of "low" dose irradiation are: 1) denuding of the germinal epithelium, 2) damage to spermatogonia, and 3) reduction of number of sperm in the ejaculate to azoospermia at the 100r dose.

To evolve these conclusions serial testicular biopsies were obtained at intervals of four to six hours, 16, 24, 40, 46, 60 and 72 days following irradiation. The germinal cells were studied by light microscopy. The first time of visualizing total depletion in the testis was after 46 days. This is exactly the period of time needed

for development of the preleptotene spermatocyte into a mature spermatozoa about to leave the Sertoli cell cytoplasm (11). The time of visualizing the first effect following irradiation was four to six hours. The germinal cells damaged at this time were the spermatogonia. The time of the first effect of X-ray as revealed by the seminal fluid was after 46 days. This is accounted for by the normal development of spermatocytes (46 days) plus the transit time (21 days) during transport of sperm through the ductular system to the ejaculate. Hence azoospermia was not revealed prior to 67 days.

The intermediate dosages of X-ray irradiation (less than 400r, greater than 100r) cause degeneration in an additional group of cells, the spermatocytes. In contrast to the lower dosages where the spermatocytes develop normally, at intermediate doses, these cells are covertly injured and not all are allowed to proceed through normal maturation-division. As a result spermatids arising from irradiated spermatocytes are decreased in number.

Spermatogonia are overtly injured and their numbers are decreased even more than at the lower doses. This ultimately results in a longer recovery time. Therefore, we have found that at intermediate dosages of between 100 and 400r:

1. Spermatogonia show overt damage but no decrease in numbers within the first 24 hours.

2. Spermatocytes are covertly affected and degenerate while proceeding through maturation-division.

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3. The result of spermatocyte injury is a significant decrease in spermatids.

4. The sperm count regularly falls to azoospermia after approximately 67 days.

5. The seminal fluid fails to reveal the reduction in normal spermatid numbers during the first 46 days. This is possibly accounted for by the time of residence of spermatozoa in the ductular system. This varies from one to 21 days (6). Thus mixing of generations of spermatozoa may obscure the reduction in testicular production of spermatozoa.

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High doses of irradiation (400 - 600r) yield a further response. All cells of the germinal series are injured. Spermatogonia and spermatocytes are overtly damaged and spermatids are covertly damaged. The additional damage to spermatids (revealed by quantitation of germinal cells and sperm count) and the overt spermatocyte damage are found only at this high irradiation dose. As a result of the severe decimation of cell numbers prior to 46 days, the sperm count also falls sharply prior to 46 days. Thus, at high dosages of irradiation, 400r and above, we have found:

1. Spermatogonia and spermatocytes are overtly damaged.

2. Spermatids are covertly damaged as revealed by quantitation of testicular cytology and sperm count and morphology.

3. Significant decreases in all cells of the germinal series are observed prior to 46 days.

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4. A significant decrease occurs in sperm count prior to 46 days.

5. The sperm count falls sharply to azoospermia and stays there many months.

6. The first visualization of spermatogonial degeneration was 16 minutes after irradiation. The observations were made ultrastructurally.

We conclude from the combination of morphological and quantitative data from all dosages that the spermatogonia are the most radiosensitive cell types and spermatids are probably the most radioresistant cells. Our conclusions are summarized in the following table:

SUMMARY OF RADIATION EFFECT UPON GERMINAL CELLS

	Spermatogonia			Spermatocytes				Spermatids			
Radiation Dose (r)	Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
10-100	+	+	+	O	O	O	O	O	O	O	O
200-300	+	+	+	⊕	⊕	⊕	⊕	O	O	O	O
400-600	++	++	++	+	+	+	+	⊕	⊕	⊕	⊕

Where: O indicates no effect, + indicates morphological change, ++ indicates greater morphological change, and ⊕ indicates possible damage but no observable morphological change, i.e., "covert" damage.

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A striking aspect of this data is that cells literally next to each other both in development and spacial placement within the tubule have such different radioresistance. For instance, the type B spermatogonium is the most radiosensitive cell. The cells preceding it (the Ad and Ap) are also radiosensitive. However, the cell arising from the B spermatogonium, the preleptotene spermatocyte, requires ten-fold the amount of X-ray irradiation that the B requires to be damaged. The next major difference in cell radiosensitivity is between the pachytene spermatocyte and the Sa spermatid. Again these cells are adjacent both developmentally and spacially, yet the spermatid requires four times the amount of radiation to be damaged. Thus the spermatids are 40 times as resistant to irradiation damage as are the spermatogonia.

In the past year we have completed all proposed irradiations of subjects and thus have an outline of the cytological defect in relation to dose and time. At present we are analyzing the effects at various dose levels in order to have statistically valid dose-response curves for histological quantitation and sperm count.

b. Objective: "To find the minimal dosage that will affect the germinal epithelium."

The foregoing discussion has revealed that cytological defects were observed at many dose levels. The minimal dose level that produces azoospermia, for example, is 100r. Marked oligospermia was produced following irradiation doses of 78r (seven subjects studied) and 50r (four subjects studied). Sperm counts were decreased from

normal to circa 2 m/cc in each of these eleven subjects. Moderate oligospermia was produced at 20r (eight subjects) and 15r (one subject).

On the basis of the effects upon sperm counts, testicular cytology, and hormonal studies, the 50 and 78r subjects can be grouped in a single dosage class, and the 15, 20, and 25r groups can be consolidated as giving a single biological effect.

Of the three receiving 10r, insufficient seminal fluid data (or none) was obtained because of: 1) sudden parole, 2) prior vasectomy and 3) one subject was subjected to too frequent biopsy operations to be certain that the fall in sperm count was indeed due to irradiation.

Of the four receiving 8r, two failed to reveal a drop in sperm count and two revealed a minimal or equivocal drop. In the latter two the drop at best was transient, however, the lowest anticipated point in time for these subjects occurred during the riots at the Oregon State Penitentiary, and no observations were possible during this critical period.

In the 8r group, testicular biopsies were avoided in order to observe "pure" seminal fluid effects. Of the three receiving 10r, only one had serial biopsies for cytological study, another had biopsies for chromosomal study only. The cytological study, however, did reveal irradiation induced changes.

Assuming "no change" in sperm count for the four subjects receiving 8r, does not rule out the possibility of transient or

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minimal cytological damage. The latter could be obscured in the seminal fluid analysis due to mixing of generations of spermatozoa during the 21-day transit time through the ductular apparatus.

The status of "the minimal dosage affecting cytology" thus is that irradiation doses as low as 10r result in damage to the germinal epithelium, in one instance, and doses of 15, 20 and 25r regularly result in damage. We now have added the additional subjects at the required dosages to study both seminal fluid observations and testicular biopsies to more clearly define the minimal dose of X-ray irradiation to the human testis.

c. Objective: "To determine the time of recovery from any given dose."

Recovery time is being determined using two parameters: sperm count and testicular cytology. The two endpoints being applied are the first appearance of spermatozoa in the seminal fluid (if the subject was at azoospermia) or the first increase noted (if the subject was oligospermic) and the first resurgence of maturation of the spermatogonial cells of the testis.

We are finding a large dichotomy in the starting time of testicular recovery versus the increase in seminal fluid sperm count, most pronounced at the intermediate and high doses. Beginning testicular recovery precedes the appearance of sperm in the seminal fluid.

The status of this aspect of the investigation is at the completion of the probing stage, which has revealed the dichotomy

mentioned above. Hence the timing of the beginning of recovery of the germinal cells is now only a rough approximation. Now that the times that biopsies should be obtained for a given dose to yield the maximal information is better understood, we can obtain precise data on recovery. The above will, of course, apply as well to the objective "spermatogonial renewal".

Two other facets of the mechanisms of the investigation upon cytological recovery have kept the number of observations minimal. These are that in approximately one-half of the subjects, biopsies have been avoided in order not to interfere with sperm counts. The second reason, is that no matter how carefully a subject is selected, from the point of view of time to be served in the penitentiary, the attrition rate after some years is extremely high. Attrition is due to unexpected pardons or parole, transfer to the Forest Camp, violation of prison regulations and removal from the general population (and from the experiment) or voluntary withdrawal. Some recovery data has been gathered from each dosage studied and is tabulated as follows:

DOSE	TIME OF BEGINNING HISTOLOGICAL RECOVERY	FIRST SPERM IN SEMINAL FLUID OR FIRST INCREASE IN NUMBER	COMPLETE RECOVERY BASED ON SEMINAL FLUID COUNTS
8r	----------	Decrease transient or non-existent	----------
10r	7 months*	2½ months	9 months
15r	----------	7½ months	16 months
20r	6 months	6 months	18 months
25r	6½ months	----------	histological only 15 months
50r	16 months*	6 months	17 months
78r	----------	10 months	----------
100r	7 months	7 months	18 months
200r	7 months	11 months	32 months
300r	7½ months	9 months	29 months
400r	10 months	none through 14 months	----------
600r	7½ months	24 months	none through 57 months

* Recovery could begin significantly earlier. Biopsies were not taken at optimal times.

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The preliminary observations suggest that at low doses (10 - 100r) beginning recovery of sperm count occurs at about six months, for intermediate doses (200 - 300r) at ten months, and for high doses (400 - 600r) at about two years.

d. Objective - Complete recovery: "To determine whether recovery is complete or incomplete. To be certain that the same level of sperm count is attained following irradiation as existed before irradiation." (not explicitly stated under I. OBJECTIVES, but often discussed with the Committee).

For each subject, many, many control seminal fluid evaluations had to be made to establish a normal value that is statistically acceptable. After the recovery phase, the same number of observations must be made to establish a value that is statistically acceptable. The preliminary result is that in those subjects where sufficient time has elapsed and sufficient observations have been available, recovery appears to be complete, i.e., the same sperm count levels have been attained.

The preliminary observations on complete recovery as seen from the table suggest that for the low doses, it is nine to 18 months, for intermediate doses, 30 months, and for high doses, longer than 57 months (none recovered to date).

e. Objective: "To determine whether subsequent irradiation following depression and full recovery will lead to a more severe reaction, postpone recovery, or lead to incomplete recovery." (another objective not stated, but discussed with, and by, Dr. Paul Henshaw)

In seven subjects that were irradiated a second or third time

following complete recovery, the response to the repeated dose was in every way comparable to the initial dose-response.

f. Objective: "To assess the problem of spermatogonial renewal in man."

The problem of spermatogonial renewal is being approached in two ways, the quantitation of the germinal epithelium during the depletion and early recovery phases, and the observation of ultrastructural changes in spermatogonia throughout the post-irradiation period.

We know from quantitation data that spermatogonial renewal in man follows a different pattern than that found in animals. For example, in the mouse, spermatogonia will completely repopulate themselves in all tubules before beginning differentiation into spermatocytes and spermatids. In man spermatogonia act in an inexplicable manner, producing spermatogonia or spermatocytes at random. Additionally, the morphology of some of the spermatogonia present during the depleted and early recovery period is different from normal morphology. Therefore they are extremely difficult to identify as A dark (Ad) or A pale (Ap) spermatogonia. A quantitative analysis of a biopsy taken during early recovery reveals that some tubules have active spermatogonia, producing spermatocytes, etc., while an adjacent tubule will be devoid of more than a single spermatogonium. All of the foregoing information suggested that additional types of spermatogonia existed, perhaps more radioresistant and with

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elongated periods between mitosis.

We have analyzed the ultrastructure of the normal spermatogonia, in preparation for the study of irradiated material, and even in normal biopsies four distinct spermatogonial types were identified (8). The fourth previously unidentified cell probably precedes the others in the developmental series and as yet cannot be identified in paraffin sections utilized in quantitation. We are now beginning to examine the ultrastructure of irradiated spermatogonia and will determine if the cell remaining after germinal cell depletion is indeed the "new" cell.

2. Cytological Accomplishments Relevant to Delineating the Limits of Normal Human Testes; New Methods of Approach and New Physiological Principles.

a. Quantitation of germinal epithelium

Quantitation of the germinal epithelium was begun early in 1964. Dr. Eugene Oakberg was instructed in the human spermatogenesis pattern and he, in turn, aided us with his extensive knowledge in quantitation of mouse germinal epithelium. After many independent trial counts by Miss Rowley and Dr. Oakberg, good agreement was reached on counting of stage boundaries, cell fragments, section edges, cell types, stage definition, and tubule cell counts. The statistical sample to be used was established for reliability at the 95% confidence level.

In August of the same year, Drs. Heller, Clermont and Oakberg

and Miss Rowley scrutinized the entire method at Dr. Yves Clermont's laboratory at McGill University. Since then, our laboratory has taken biopsies at selected times after x-irradiation for quantitation of damage. A vast bulk of control data has also been collected and has revealed a great amount of further information on normal spermatogenesis. Dr. Oakberg has independently confirmed counts on many biopsies previously counted at the Foundation.

Man is unique among mammals in having small clusters of areas comprising a given cell association rather than having an entire section of tubular cross-section and length involved in one cell association. Thus, in a given cross-section of a seminiferous tubule three to five different cell associations may be encountered. Quantitation, therefore, was approached in two ways (5): (1) counting cells per cell association, and (2) counting cells per tubular cross-section without regard to the cell associations involved. The latter method proved to be important as results of the effect of radiation of testes in man emerged. These results revealed that during the process of depletion and during the early recovery period, the landmarks defining the cell associations were obliterated.

In both counting methods, biopsy sections were scanned in a manner similar to blood smears. If one section was not of a sufficient size to collect a statistical sample, another section 40μ or more away from the first would be counted. The slides to be used for confirmation counts at Oak Ridge were always at the opposite end of

the series of serial sections and thereby separated by as many as 500μ from those sections counted in Seattle.

The main criterion used for the selection of a tubule to be counted was the angle of the cut. Tangential cuts were not counted. Since the cells mature from basement membrane to lumen, only tubules or areas in which the cells would have matured in the plane of the slice were used. Tubules with artifacts or unidentifiable lumens were not counted. In both methods, it was found that either 30 tubular cross-sections or 30 cellular associations gave statistically reliable results ($P < 0.05$).

Forty control subjects were analyzed by quantitating the germinal cells according to their cell associations. The results for each cell type were expressed as germinal cell/Sertoli cell ratios. The Sertoli cell was selected as the stable standard against which to express and compare cell numbers since under the conditions of the experiment, changes in germinal cell numbers and tubular length and diameter could be expected, but Sertoli cells were expected to remain constant. The means for each cell type/Sertoli cell ratio and their standard deviations and standard errors are as follows:

Cell Type	Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
Mean	0.65	0.59	0.37	0.27	0.64	0.08	2.74	1.68	2.19	1.81	2.27
S.D. ±	0.10	0.11	0.13	0.11	0.21	0.05	0.43	0.30	0.54	0.59	0.68
S.E. ±	0.02	0.02	0.02	0.02	0.03	0.01	0.07	0.05	0.09	0.09	0.11

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These ratios were then compared with quantitation of 25 control subjects (often the same subject) by counting the germinal cells in cross-sections of tubules without regard to the cell association. The means for each cell type/Sertoli cell ratio and their standard deviations and standard errors are as follows:

Cell Type	Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
Mean	0.51	0.49	0.26	0.19	0.18	0.07	2.64	1.61	1.24	1.27	1.01
S.D. ±	0.12	0.11	0.05	0.07	0.08	0.03	0.47	0.53	0.36	0.31	0.30
S.E. ±	0.02	0.02	0.01	0.01	0.02	0.01	0.09	0.11	0.07	0.06	0.06

Good agreement between these two approaches was found. Thus, the latter method of quantitating control subjects allowed for comparisons of serial testicular biopsies following exposure to radiation. Note the cell association counts reveal a higher number of leptotene spermatocytes (L) than the tubule counts. This difference in leptotene counts has been discussed with Dr. Yves Clermont of McGill University and a tentative explanation is offered. It is possible that the initial timing of the duration of this cell type was slightly incorrect. If, indeed, the pre-leptotene did last a short time in cell association IV and the leptotene changed to zygotene shortly before the end of cell association V then the tubule method would have fewer leptotenes than the cell association method. The cell association method requires leptotenes to be present and pre-leptotenes and zygotenes to be absent before cell associations IV and V can be counted.

Quantitative analysis has been completed on many subjects irradiated. This procedure involves obtaining testicular biopsy specimens at various intervals after the time of irradiation and using the procedures previously described. Examples of such analyses for three subjects receiving 25, 100, or 600r are summarized in Table I.

With these hypotheses in mind we need to subject the data of the quantitation to careful statistical analysis. From such an analysis, appropriate groupings of times after irradiation and doses of radiation may be made. In turn, this should set guidelines for determining how many additional subjects are required for each grouping to establish a statistically significant result. Having fulfilled these prerequisites a dose-response curve for the damage and depletion of each cell type can be constructed.

A careful analysis of the ultrastructure of the spermatocytes exposed to 100, 200 and 300r may well reveal cytological alterations that remain undetected by the light microscope. We are now collecting and analyzing material for such examination.

b. Ultrastructural studies

Utilizing the electron microscope, we have succeeded in identifying and describing the ultrastructure of four types of spermatogonia (8). Previously only three spermatogonial types were known. The use of 1μ thick Epon sections with light microscopy afforded visualization of considerably more cytological

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TABLE I.

	Ad	Ap	B	R	L	Z	P	Sa	Sb	Sc	Sd
	Spermatogonia			Spermatocytes				Spermatids			
Control	0.93	0.80	0.49	0.42	0.76	0.11	2.56	1.56	3.09	2.67	2.45
25r											
6hrs	0.68	0.63	0.25	0.09	0.45	0.01	2.87	1.78	2.62	1.13	1.54
24days	0.74	0.64	0.29	0.05	0.14	0.02	2.85	2.65	2.00	0.27	0.35
40days	0.28	0.23	0.03	0.02	0.04	0	0.20	0.30		1.16	
201days	0.36	0.28	0.07	0.03	0.04	0	0.31	0.21		0.41	
420days	0.98	0.88	0.30	0.45	0.25	0.20	3.31	0.99	2.45	3.49	
Control	0.71	0.53	0.28	0.24	0.43	0.06	1.99	1.43	1.29	1.29	1.00
100r											
24hrs	0.62	0.44	0.14	0.14	0.33	0.02	1.32	0.72	0.79	0.71	0.74
14days	0.59	0.06	0.003	0	0.009	0	1.05	0.55	0.59	0.73	0.49
[illegible]ys	0.20	0.08	0.04	0.02	0	0	0.27	0.81	1.06	0.75	0.73
49days	0.27	0.12	0.02	0.03	0.04	0	0.20	0.17		0.32	
112days	0.06	0.02	0	0	0	0	0.03	0.02		0.05	
210days	0.06	0.06	0.005	0	0	0	0.06	0.03		0.02	
Control	0.56	0.44	0.29	0.33	0.61	0.01	2.60	1.58	2.20	1.83	1.60
600r											
22hrs	0.47	0.24	0.07	0.07	0.74	0.04	2.90	1.88	2.24	2.22	1.68
14days	0.28	0.23	0.004	0	0.01	0	1.39	0.44	0.99	0.71	0.72
29days	0.21	0.14	0.01	0	0	0	0.01	1.34		2.37	
84days	0.01	0.002	0	0	0	0	0	0	0	0	0
151days	0.002	0	0	0	0	0	0	0	0	0	0
[illegible]ys	0.006	0	0	0	0	0	0	0	0	0	0
32[illegible]days	0.003	0.01	0.02	0.005	0	0	0.05	0.04		0.01	
477days	0.001	0	0	0.006	0	0	0.01	0	0	0	0

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detail than can be obtained with paraffin-embedded material, and, consequently, four distinct types of spermatogonia could be identified in such Epon sections. However, further study of the Epon sections with electron microscopy provided the most satisfactory method for revealing different spermatogonial types. This method revealed a distinct difference in the ultrastructural characteristics of the four types of human spermatogonia. In addition, previously undescribed structures were found to occur only in certain spermatogonia. We are using the same method to study the normal morphology of the more mature cell types and the effect of X-ray irradiation on the spermatogonia.

Basically, we did 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. Secondly, we studied Epon-embedded tissues sectioned at micron or sub-micron thicknesses by light microscopy and identified the spermatogonial types by comparing these cells with the paraffin-embedded material. Thirdly, electron microscopic examination of thin sections adjacent to the sections used for light microscopic examination was used to determine the ultrastructural characteristics of the different types of spermatogonia. In this way we were assured that the cells we called Ad, Ap and B spermatogonia using paraffin-embedded

tissue with the light microscope would correspond to the cells we term Ad, Ap and B spermatogonia using Epon-embedded material with either the light or electron microscope. We propose to continue this three-pronged approach to the study of the other cells of the germinal series.

Four spermatogonial types were identified, the AL, Ad, Ap and B. The AL (L for length of cell on basal lamina) is considered to be an earlier cell than the Ad. The mitochondria of all four spermatogonial types do not have a plentiful supply of cristae. This condition of few cristae being present is generally correlated with inactive cells and related to low energy requirements [12]. 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, can be interpreted as a morphological manifestation of a mechanism that allows a short diffusion path for ATP that is required for some function in the endoplasmic reticulum. André [13] has suggested that a close mitochondria to endoplasmic reticulum association might serve to supply energy for protein synthesis.

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 to ribosomes and, while we hasten to repeat that the function of the filamentous body is unknown, it is possible that this structure may represent a highly modified mechanism for protein synthesis.

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 pinocytotic vesicles occur in these cells our evidence is adequate enough to discount the movement of material in these vesicles in a reverse pinocytotic direction. One interesting facet of these pinocytotic vesicles is that they occur only on adjacent cell-cell surfaces and not on the spermatogonial surface next to the basal lamina.

An increased number of myelin-like arrays, thought to represent phospholipid degeneration, were present in the Ap and B spermatogonia, as compared to the absence of such structures in the AL and Ad. spermatogonia. This suggests that autolysis occurs in the more mature spermatogonia.

The Ad spermatogonium has been considered to be the most primitive spermatogonial type [14]. Clermont [15] suggests that the Ad spermatogonium has an "equivalent" mitosis and gives rise to either two Ad spermatogonia or to two Ap spermatogonia. Each Ap

spermatogonium subsequently gives rise to two type B spermatogonia which then give rise to the spermatocytes, the concept being that the Ad represents a cell that can either perpetuate the primitive stem cells or give rise to a spermatogonium, i.e., the Ap spermatogonium, that is committed to spermatogenesis. Our findings support and extend the portion of Clermont's work that suggests that there is a primitive spermatogonium, an intermediate form, and an advanced spermatogonium.

Further, we have found instances of binucleate AL, Ad and Ap spermatogonia and we interpret these cells as having completed karyokinesis and of being in the process of completing cytokinesis. 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 divides mitotically to form two of the next more mature spermatogonia.

On the basis of our evidence we cannot rule out the possibility of differentiation of any particular spermatogonial type into the next spermatogonial type to explain the origin of successive spermatogonia. If this were the case, however, one would expect to find intermediate forms and such intermediate forms have not been observed.

The correlation between our findings and Clermont's (15) 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 spermatogonium represents a primitive spermatogonium that is capable of either regeneration of the stem cell population or giving rise to the Ad spermatogonia which then form the Ap spermatogonia, etc.? In this situation, the Ad spermatogonium would be the earliest spermatogonium committed to spermatogenesis. This possibility can be subjected to experimentation by depleting the spermatogonial population, not to mention the spermatid population, by irradiation and examining the tubule for remaining spermatogonia that must persist in order to give rise to spermatids by "recovery".

Details of the methods and the cell descriptions are covered in the publication by Rowley, Berlin and Heller (8).

Ultrastructural examination of the germinal epithelium following irradiation has revealed many morphological changes, some of which we have not seen in either normal or otherwise altered testes. For example, at 24 days after irradiation at 600r, we have found macrophages traveling through the basal lamina into the tubules. We believe they pick up damaged cellular material and then move into the lumen. The Sertoli cells, however, also act as disposals for damaged tissue releasing acid phosphatase into the cell to break it up and then acting as a transporting medium for the broken-up cells to pass through into the lumen.

We are currently still examining these biopsies for additional changes. Although much progress has been made we have really only

scratched the surface. Because of the number of biopsies taken and the wealth of information available in each biopsy, much of the material has not been examined as yet.

c. Seminal fluid examination

i) Effect of biopsy upon sperm count: Man is unique in his capability of being able to undergo a single biopsy, or even several testicular biopsies over a period of time. This is not generally true for other animals. Thus man is an ideal experimental subject from this point of view, as comparisons between testicular biopsy and sperm counts can be made. However, the effect of the biopsy procedure on the seminal fluid concentration was previously unknown. Because we followed both parameters in all of our irradiation studies (as well as other experimental situations), we examined their interrelationships in normal control subjects. Of the subjects analyzed, only 39% experienced a significant ($P \leq 0.05$) drop in sperm count following biopsy. Of those subjects with multiple biopsies, 27% showed a decrease after the last biopsy procedure. All sperm count decreases occurred in the first ten weeks after biopsy and all subjects showed complete recovery by 18 weeks. Our conclusions were that ten to 18 weeks should elapse following a biopsy before initiating therapy or experimental treatment. These data have been reported by Rowley and Heller [16] and Rowley, et al., [4].

In designing our irradiation experiments, we have used this information as follows. All subjects had a control biopsy. They were allowed ten to 18 weeks to recover from the biopsy effect before irradiation.

Our subjects were divided into two groups, one specifically for the study of seminal fluid and hormone studies without biopsies and the other for biopsy studies along with seminal fluid examination and hormone evaluation. In this way we were able to safeguard against confusion of changes caused by biopsy with actual changes caused by X-ray.

ii) Duration of transport of spermatozoa through the ductular system: X-ray irradiation has also been used to extend our basic knowledge of the process of spermatogenesis by determining the amount of time required by sperm to pass through the ductular system of the male. Testes of normal men were exposed to single doses of ionizing radiation or injected with tritiated thymidine. The selected X-ray dose was sufficient to cause degeneration of spermatogonia while allowing each of the other germinal cells to mature normally. The pre-leptotene spermatocyte (the least advanced unaffected cell) is released into the tubular lumen 46 days following irradiation. Since spermatozoa were absent from the seminal fluid 67 days after irradiation, the longest travel time of a sperm discharged into the tubular lumen is three weeks (67 - 46 = 21 days).

A second group of men was given intratesticular injections of H^3 thymidine. Seminal fluid for autoradiography was collected every third day. The most advanced cell labeled within one hour is the preleptotene spermatocyte. This is released into the lumen of the tubule as a mature sperm 46 days after labeling. Counts of labeled spermatozoa indicate that the earliest sperm appeared in the ejaculate 46 or 47 days after

injection. The greatest number appeared in the ejaculate in 54 to 56 days. Combining irradiation and thymidine data we conclude that the duration of transport of spermatozoa in man varies from one to 21 days with the majority of the spermatozoa appearing in the ejaculate ten to 14 days after release from the Sertoli cell cytoplasm. These results have been reported by Heller, et al. (6).

iii) Seminal fluid counts: Interesting results have been obtained from studying the changes in seminal fluid after X-ray irradiation. Those subjects receiving doses below 50r (8r, 10r, 15r, 20r and 25r), maintained a sperm count of 10 m/cc or higher. A dose of 50r or 78r brought the count to a level of about 1 m/cc. One hundred roentgens appears to be a threshold dose as some subjects attained total azoospermia while others maintained counts of about 0.2 m/cc. All subjects receiving doses above 100r went to complete azoospermia for extended periods of time.

Very high doses (400 - 600r) had a different effect on seminal fluid counts than did the lower doses. The counts show a striking drop below control levels before the 46-day period is complete. This pre-46-day drop in sperm numbers indicates immediate destruction of spermatocytes as well as spermatogonia. Doses below 400r show immediate destruction of spermatogonia only. Preliminary results were reported by Heller, et al. (17).

Recovery times vary with the individual and with the dose of radiation given. Below 100r, the beginning of recovery is not dose-dependent. Attaining a normal recovery sperm count appears to be

more dose-dependent.

iv) Sperm morphology: Variations in sperm morphology have been studied by MacLeod and he has reported both the variation of shapes and the percentage of each shape found in normal ejaculates [18]. Dr. MacLeod (Cornell Medical School) has examined serial seminal fluid samples from our radiation subjects and counted abnormal forms. The percentage of normal sperm dropped negligibly after 15r and 100r. With 600r, a dose that produces persistent azoospermia, more dramatic changes are observed.

d. Leydig cells

An objective method to reflect changes in Leydig cell number which might occur as their activity was altered has long been needed. Because the absolute number of Leydig cells in the testis cannot be counted, it is necessary to relate the number of Leydig cells present in a given area to some constant structure in the testis. The Sertoli cells have been chosen as an index of the number of Leydig cells present since their number is not altered by treatment with drugs or hormones, they do not divide in the adult [19,20], and they have been widely accepted by other investigators as a constant for quantitation [21, 22].

Our quantitation method [7] involves taking numerous photomicrographs of serial sections throughout a biopsy using a 10X objective. These photomicrographs are then used as a guide for counting all the Sertoli cell and Leydig cell nucleoli within the photographed area

using 400X magnification. The results of these counts are expressed as a ratio of Leydig cells to Sertoli cells (LC/SC). The section thickness may vary slightly, the tubules may shrink or the germinal cells may be depleted as a result of treatment, but the LC/SC ratios in control and treatment biopsies will still be directly comparable since the method compensates for such changes.

To test the method we gave human chorionic gonadotropin (HCG) to six normal men, observed a several-fold rise in urinary testosterone, and looked for a change in the number, mass or morphology of the Leydig cells [23]. An increase in number of Leydig cells was expected because Maddock and Nelson [24] had reported such an increase in abnormal and normal males treated with chorionic gonadotropin. Their report was based on a subjective impression from biopsy sections. We used our method to quantitate Leydig cells before and after treatment to see if an actual increase in number was occurring. Since HCG causes shrinkage of the tubules from depletion of the germinal epithelium, the subjectively observed increase by Maddock and Nelson might have been due to the crowding of the Leydig cells into a more compact interstitial area (since the tunica propria is elastic).

The following table summarizes the results of administration of HCG to six normal men on the number of Leydig cells:

SUBJECT	LC/SC ± s.e. CONTROL	LC/SC ± s.e. 6 WKS. HCG	LC/SC ± s.e. 16 WKS. HCG
#1	0.56 ± 0.02	0.57 ± 0.02*	
#2	0.27 ± 0.01	0.21 ± 0.02Δ	
#3	0.37 ± 0.01	0.43 ± 0.02▲	
#4	0.67 ± 0.03	0.71 ± 0.02*	0.59 ± 0.02Δ
#5	0.72 ± 0.02		0.77 ± 0.02*
#6 (rt. testis)	0.42 ± 0.03		0.49 ± 0.02*
(lft. testis)	0.50 ± 0.03		0.52 ± 0.02*

* no significant change

Δ statistically significant decrease

▲ statistically significant increase

The Student's "T" test was used to analyze the data ($P \leq 0.05$). After six weeks of administration, two subjects showed no change, one increased and one decreased. Leydig cell ratios after 16 weeks revealed that two subjects showed no change and one subject decreased. These results indicate that the changes were random and therefore administration of HCG caused no significant increase in the numbers of Leydig cells, and furthermore, that the increases reported by Maddock and Nelson were visual changes not substantiated by our quantitative method.

After the validity of the procedure was demonstrated by the HCG experiment, we have begun to apply the Leydig cell method to material collected at various times after irradiation. Data from one subject irradiated with 600r is presented in the following table:

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SUBJECT V199

TIME AFTER IRRADIATION	RIGHT TESTIS LC/SC RATIO ± s.e.	LEFT TESTIS LC/SC RATIO ± s.e.
CONTROL	0.45 ± 0.03	0.47 ± 0.02
26 days	0.44 ± 0.03*	
92 days	0.53 ± 0.02▲	
232 days		0.52 ± 0.03▲
295 days	0.63 ± 0.03▲	
1022 days	0.55 ± 0.04▲	
1120 days		0.75 ± 0.05▲
1253 days	0.49 ± 0.02*	
1722 days		0.88 ± 0.03▲
1904 days		0.61 ± 0.03▲

* no significant change

▲ significant increase above control

We observed a significant increase in the LC/SC ratio by 92 days following irradiation. This increase has been observed through 1722 days post-irradiation. A drop occurs at 1253 days for the right testis and at 1904 for the left testis, suggesting a recovery of Leydig cells, substantiated by the fact that the germinal epithelium is in early recovery, which together, would indicate that the testis is in the process of functional recovery. Thus far four subjects quantitated show this increase after irradiation with 600r.

Although a total of 15 subjects have received 600r of irradiation only 12 have sufficient biopsies taken after 90 days to ascertain if

an increase has taken place. Only one subject (mentioned above) has been followed for a long enough time to begin to show a recovery in Leydig cell numbers. The number of men that will be available after this amount of time has elapsed can only be postulated. At this time seven subjects who have received 600r are actively participating in the irradiation program. Three subjects have biopsies after 1000 days post-irradiation and the rest have biopsies after 360 days of irradiation. We shall continue to follow these subjects for as long as possible. Hopefully until they reach control levels. Should we find significant changes in all these subjects at 600r irradiation, we are prepared to investigate subjects at lower doses of irradiation. Although the task of quantitating these numerous biopsies involves hours of a technician's time, they should yield valid data as to the effect of irradiation on Leydig cell numbers.

We have been examining and characterizing the ultrastructural features of Leydig cells in control biopsies before carefully examining those after irradiation. Very preliminary examination indicates Leydig cells are affected by irradiation within two days. This study will enable us to determine morphological changes of the Leydig cells which cannot be seen with the light microscope We will attempt to answer questions concerning specific effects of x-ray on these cells and determine if there is a dose-response relationship involved.

3. Hormonal Objectives

To determine the influence of any given radiation-produced testicular alteration upon other parameters such as: a) urinary

total gonadotropins, b) urinary interstitial cell-stimulating hormone (ICSH), c) urinary follicle-stimulating hormone (FSH), d) plasma FSH, e) urinary estrogens, f) urinary testosterone and epi-testosterone, g) plasma ICSH, and h) plasma testosterone.

a. In each subject and for any dose level of irradiation, a distinct rise in urinary gonadotropins was noted. The rise occurred concomitantly with the first denuding of the germinal epithelium. The rise was sustained until histological recovery began. Subsequent lowering to normal levels paralleled the repopulation of the seminiferous tubules.

Our explanation for the rise in gonadotropins is that the germinal cells, during their normal course of maturation and development, require gonadotropins. In the absence of germinal cell activity no utilization occurs and consequently excess gonadotropins appear in the urine. The lowering of gonadotropins occurs as the germinal cells again become active during the recovery period. Since the gonadotropin assay is a measure of both FSH and ICSH, it became necessary to determine which gonadotropin was mainly involved.

b. Measuring urinary ICSH on the same urine samples revealed no change following any irradiation exposure at any time during the course of depletion, quiescent period, or during germinal epithelial recovery.

c. Measuring urinary FSH revealed a rise during depletion, sustained during the quiescent period and a lowering to normal during recovery. This in every way paralleled the rise and fall of the total gonadotropins. Our interpretation is therefore that the

rise of total gonadotropins is due to the rise in FSH and that the germinal cells, during their period of activity, utilize FSH.

At this time data is being analyzed to give statistical significance to these observations. The results appear to confirm the above. Four subjects on 8r revealed no change in FSH and presumably little or no change in germinal epithelial function as inferred from sperm counts. Those subjects at the higher doses with drops in sperm counts revealed the rise.

d. Measuring plasma FSH confirmed those results obtained from the urine. No change was found in plasma FSH at 8r, a slight increase was seen at 20r and highly significant increases were seen at 78 - 600r. At these dosages plasma FSH rose as much as four-fold.

In order to evaluate Leydig cell function following irradiation, studies of estrogen, testosterone and epitestosterone excretion and plasma ICSH were observed.

e. Estrogen excretion can be used as a measure of Leydig cell function for the human male (24). This relatively insensitive method failed to reveal any change. Later urinary testosterone evaluation became available and this was substituted for the estrogen assay.

f. Urinary testosterone measured by gas-liquid chromatography, revealed a minimal but statistically significant lowering following irradiation. In 1968 we changed methodology and

began using a new technique for urinary testosterone glucuronide in which testosterone and epitestosterone are determined separately (25). Because of the great variation in results we have discontinued measuring urinary testosterone and are now concentrating on the plasma testosterone assay.

g. Measuring plasma ICSH by radioimmunoassay has produced surprising results in contrast to those obtained from the urine. Only two dosages (8r and 20r) revealed no change in plasma ICSH following irradiation. At the higher doses (78r, 200r and 600r) there were definite increases in plasma ICSH. This increase was greatest at 600r where plasma ICSH values were double the control values.

The fall in urinary testosterone and the concomitant rise in ICSH strongly suggests that irradiation is causing Leydig cell failure. Preliminary observations from Leydig cell quantitation reveal an increase in Leydig cells (vide supra). It is suggested that this increase in Leydig cell numbers is an effort to compensate for their failure in function.

h. We are just beginning to obtain results from plasma testosterone determinations using a competitive protein binding method. The normal male control value obtained in this laboratory (36 plasma samples) is 515 $\pm$ 164 (S.D.) ng/100ml with a range of 212 - 896 ng/100ml. Preliminary data from two subjects at 600r and two subjects at 200r indicate no statistical change in plasma testosterone following irradiation. More samples are now being assayed at each dose level (20r, 78r, 200r and 600r) to determine if irradiation has any effect upon plasma testosterone.

4. Hormonal Accomplishments Relevant to Delineating Normal Human Testicular Physiology; Methods Used and New Methods of Approach.

a. Total gonadotropins

All urinary gonadotropins are extracted by the Kaolin-Acetone procedure described by Albert [26]. The precipitate obtained thereby is them assayed separately for total gonadotropin, FSH, and ICSH activity.

Urinary total gonadotropins were assayed in each subject at all irradiation doses. The ovarian weights of immature rats were used as the end point. These weights were expressed in milligrams of a standard, P-22-C, which caused a parallel increase in ovarian weight in the assay animal.

b. Urinary interstitial cell-stimulating hormone

Urinary ICSH was measured using the hypophysectomized rat ventral prostate bioassay devised by Greep, et al. [27]. A complete statistical evaluation of the urinary ICSH bioassay data, using the Thorslund-Paulsen parallel-line technique [28] for estimating potency was carried out using the IBM 7040 and 7094 computer facilities at the University of Washington. Urinary ICSH data is expressed in milligram equivalents of a Pergonal reference preparation.

The ICSH control data have been analyzed and yield a mean of 0.69 mg of Pergonal and a standard deviation equal to 0.10. The distribution function may be approximated by a normal curve ($\bar{X} = 0.69$, s.d. = 0.1).

c. Urinary follicle-stimulating hormone (FSH)

Urinary FSH was measured by the ovarian weight augmentation technique (1). A complete statistical evaluation of the urinary FSH bioassay data using the parallel-line technique for estimating potency was carried out with the use of a Fortran II program and the Computer Center of the University of Washington. Urinary FSH is estimated in terms of a standard Pergonal reference preparation. (28)

In 1968 all FSH data was re-analyzed using the Thorslund-Paulsen parallel-line program. The results are almost identical with those obtained using the slope-ratio technique, however, additional information was obtained concerning the internal consistency of the assays (λ, Finney's g, F-tests). For normal, healthy men, our laboratory control values have a mean of 0.11 mg P26e/24hr and a standard deviation of 0.04. The frequency distribution of scores may be approximated by a normal distribution; mean = 0.11 and standard deviation = 0.04. A goodness of fit test yielded $X^2 = 12.5$, which is less than the X^2 at 0.05 and 6 degrees of freedom. We are now in the process of re-analyzing the vast amount of urinary FSH and ICSH data we have accumulated. Each assay is being carefully evaluated both personally and with the aid of the computer. This analysis will continue for some time because of the time involved in evaluating each response that has been obtained in all subjects who received irradiation. After this careful analysis has been completed it will be inspected further to determine if there is a dose-response.

d. Urinary testosterone

The urinary testosterone method we formerly used measured testosterone and its epimer, epitestosterone, combined. This method involves enzyme hydrolysis of the testosterone glucuronide in the urine, solvent extraction, and washing to remove polar contaminants. The sample was further purified by thin-layer and paper chromatography, and the testosterone-containing zones on the chromatograms were located with a radiochromatogram scanner. The testosterone fraction was quantitated using gas-liquid chromatography by comparing the detector response of the sample testosterone to that of standard testosterone. Losses during processing (mean recovery for 900 analyses = 59%) were evaluated by addition of tracer amounts of tritiated testosterone to each urine sample and counting a one-tenth aliquot of the resultant testosterone fraction.

In 1968 we changed methodology and used a new and more specific technique for urinary testosterone glucuronide in which testosterone and epitestosterone were determined separately (25). It was carried out at The Swedish Hospital Medical Center Department of Pathology under the direction of Mr. Arthur Olson. However, as mentioned before, we have discontinued analyzing urinary testosterone in favor of plasma testosterone.

e. Plasma FSH and ICSH

The radioimmunoassays of human plasma ICSH and FSH are based on the competition between labeled and unlabeled antigen sites on specific antibodies. The importance of this assay procedure resides in

its ability to specifically measure protein quantities in the range of ng (10^{-9}g) or pg (10^{-12}g) which is 500 - 1000 times more sensitive than bioassay procedures. It also enables us to measure a large number of samples at once using small volumes of plasma or serum.

All reactants are diluted in the following buffer: 0.01 M phosphate, 0.15 M NaCl, 2% normal rabbit serum, 0.02% merthiolate, pH 7.8. The reagents for radioimmunoassay are supplied by the National Pituitary Agency and the Endocrine Section of the National Institute of Arthritis and Metabolic Diseases and include rabbit anti-HCG, LH-LER-960 (for labeling), FSH-869-2, rabbit anti-FSH, and ICSH and FSH pituitary reference preparation LER 907.

The iodination procedure is that described by Greenwood and Hunter (29). Carrier free ^{131}I is obtained in approximately 2 mc amounts in plastic polyvials from the Isoserve Corporation, Cambridge, Massachusetts. The following reagents are then added to the vial using standard preparations for handling large amounts of radioactive iodine:

1. 25λ of a 0.4 M PO4, pH 7.5.
2. 10λ of 2 μg of ICSH or FSH in a 0.1 M PO4, 0.15 M NaCl, pH 7.8 buffer.
3. 10λ of chloramine I (25 mg in 10 ml of above buffer).
4. 25λ of Na2 S205 (25 mg in 10 ml of above buffer).

The entire reaction mixture is applied to the exposed surface of the Sephadex G-75 column. The column is prepared in a soft glass tube previously equilibrated in 0.01 M PO4 NaCl, pH 7.8 at room temperature, and washed with 1 ml of 2% bovine serum albumin to coat the glassware

and Sephadex with albumin, thereby preventing absorption of radio-iodinated hormone onto the glass tube. Separation of ICSH-^{131}I or FSH-^{131}I from inorganic ^{131}I is achieved by passing this solution through the Sephadex column. The eluants are collected ten drops per tube for 20 tubes, and counted in the gamma counter. In our previous studies, two peaks of radioactivity are obtained; an early peak beginning at tube three to five and trailing off by tube six; and a second peak containing free ^{131}I beginning at about tube seven. The ICSH-^{131}I or FSH-^{131}I is contained in the first peak and generally the tube high on the trailing edge of this peak contains the least damaged ICSH-^{131}I or FSH-^{131}I and is used in the assay. The specific activity of ICSH-^{131}I is between 200 to 500 μc per μg, and is 250 to 650 μc per μg for FSH-^{131}I. (30, 31)

The radioimmunoassays will be carried out using Odell's methods with slight modifications. All reagents will be added to 10 x 75 mm tubes in the following order:

1. buffer (as mentioned earlier) to make a total volume of 1.0 ml.
2. 100λ (μl) of 0.1 MEDTA, pH 7.8.
3. 200λ of plasma to be assayed (or of "standard hormone").
4. 100λ containing 0.05 to 0.15 mμg ICSH-^{131}I or FSH-^{131}I.
5. 100λ of antisera suitably diluted usually 1:10,000 (final dilution 1:100,000 for anti-HCG) or 1:40,000 (final dilution 1:400,000 for anti-FSH).

Complete standard dose-response curves are run in all assays, and

plotted on a semilog paper. For this purpose known amounts of ICSH or FSH are added instead of plasma. The range of standard we are using is from 0.1 mIU to 100 mIU (1 mIU = 2.08 mμg of LER-907 for ICSH and 5.0 mμg for FSH). All tubes are incubated for four days at 4°C at which time 50λ of anti-RGG (2nd antibody) is added to each tube and the mixture incubated 24 hours longer at 4°C to achieve separation of antibody-bound from free ICSH-^{131}I or FSH-^{131}I. Tubes are then centrifuged at 500 g and the supernatant removed by suction. Radioactivity is measured in a gamma spectrometer, and all results expressed as a per cent of counts per precipitate. Zero per cent is defined as no ICSH-^{131}I (FSH-^{131}I in the case of FSH) bound to antibody. In our previous studies, 5 - 10% of iodinated hormone was non-specifically trapped in the precipitate. These counts could be removed by washing the precipitate but this was found not to contribute significantly to the precision of the assay. One hundred per cent is defined as the number of counts precipitated in tubes containing ICSH-^{131}I or FSH-^{131}I and antibody, but no unknown or standard ICSH or FSH. The results are calculated in terms of μg of LER-907 per 100 ml of plasma. Each assay of pooled plasma is run as a control reference along with the unknown plasma.

The plasma samples of the same subject are run in the same assay if possible. We have found that the intra-assay variation (2 S.D.) is ± 11% for ICSH and ± 10% for FSH. The inter-assay variation (2 S.D.) is ± 31% for ICSH and ± 30% for FSH. These techniques are capable of

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measuring between 0.8 ng to 30 ng per assay tube for ICSH and between 4 ng to 75 ng per assay tube for FSH. We have found that the average concentration of plasma ICSH taken from 50 healthy men is 9.04 ± 2.65 μg LER-907/100ml. The average concentration of plasma FSH taken from 35 healthy men is 34.4 ± 8.55 μg LER-907/100ml.

Since standard LER-907 was suggested by the National Pituitary Agency in 1968, two laboratories we have compared our results with are Nankin (32) and Paulsen (33). Our results are comparable to these two laboratories in terms of μg LER-907/100ml plasma, as shown in the following table:

	No. normal men	Plasma ICSH* mean	range	No. normal men	Plasma FSH mean	range
Our laboratory	50	9.0	4.8 - 15.3	35	34.0	22 - 45.
Nankin (32)	79	7.6	3 - 21.0	57	19.0	8 - 41.
Paulsen (33)	-	-	-	54	-	23 - 53

* μg LER-907/100ml plasma

We have also exchanged blinded samples with Dr. Paulsen and found equivalent values.

Our previous results are also comparable to mIU 2nd IRP-HMG if conversion factors are used. However, there is confusion if mIU of LER-907 is used, because other conversion factors are involved of which, unfortunately, many investigators are not aware. (radioimmunoassay 1 mIU LER-

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= 4.6 mIU 2nd IRP-HMG for ICSH, and 1 mIU LER-907 = 1.9 mIU 2nd IRP-HMG for FSH).

In addition, we have also measured plasma ICSH and FSH in men exhibiting a variety of abnormal conditions. A few of these are listed in the following table:

	Plasma ICSH* µg LER-907/100ml	Plasma FSH* µg LER-907/100ml
Hypogonadotropic hypogonadism	4.0	17.5
Functional prepuberal castrate syndrome	65.5	222.0
Surgical castrate	52.6	111.5
Sertoli-cell-only syndrome	26.6	126.5
Postmenopausal syndrome	121.3	436.0
Male climacteric syndrome	114.0	194.0

* Normal men (ICSH range 4.8 - 15.3; FSH range 22.0 - 45.4)

f. Plasma testosterone

Plasma testosterone is measured by competitive protein binding by the method of Murphy (34). This involves extraction of plasma with ether three times, shaking each time on a Vortex mixer. The ether phase is dried down in a water bath at 45°C with filtered air and the extract then placed on a column chromatograph of Sephadex LH-20

(column dimensions approximately 3/8" x 17"). The solvent system is chloroform:heptane:ethanol:water in the proportions 50:50:1:0.12 (saturation). The testosterone fractions, located by running an identical column of tritium-labeled testosterone only, are collected in 2.5 ml aliquots and dried down.

To determine the amount of testosterone in these fractions, standard curves are set up for each assay. A series of tubes containing 0, .1, .2, .4, .6, .8, 1.0, 1.2 and 1.5 ng of testosterone in ethanol are dried down. To these standard curve tubes and the fractions containing unknown amounts of testosterone are added .1 ml of "protein tracer" (0.1 ml third trimester pregnancy plasma, 0.09 ml of 10 μc/ml ^{3}H-T in ethanol, 10 ml .2 M phosphate buffer). The tubes are then incubated five minutes at 45°C, then 30 minutes at 4°C. All following procedures are carried out at 4°C. A 1 ml aliquot of 10% Korenman's suspension (in phosphate buffer) is then added to each tube to adsorb free testosterone, and the tubes incubated for five minutes. The tubes are shaken for one minute, then centrifuged at 4000 rpm for five minutes. A 0.5 ml aliquot of the supernatant is pipetted off and counted in a scintillation counter. The curve obtained by plotting cpm vs. ng testosterone in the standard curve tubes is used to determine the amount of testosterone in the plasma sample. Results are reported as ng testosterone/100ml plasma.

This assay can be performed by two persons in two and one-half days, running 14 columns - six duplicate samples, one tracer column, and one plasma pool column. We found that all glassware must be washed ultrasonically, then washed with distilled ethanol, and finally washed with double-distilled H_2O. Ether used in the extraction must be freshly

distilled. We find we can run three assays every two weeks, with fortuitous timing of dishwashing, distillations, etc. We currently do not distill the solvents in our solvent system; instead, we treat the solvent system with Norit A and filter through a Millipore system.

One of the biggest problems in all plasma testosterone assays by competitive protein binding has been the solvent blank (34, 35) in the chromatography procedures. It affects the amount of testosterone "seen" by the protein - either by competing with testosterone or somehow altering the site of binding. There is still much discussion on the mechanism of this effect. Besides presenting problems for the standard curve, the solvent blank is often not reproducible.

It has been suggested that the effect of the solvent blank be "subtracted" from the standard curve. This is not possible, as the solvent blank effect varies with the level of testosterone (34, 35).

This method eliminates both problems. Chromatography on LH-20 presents a small and reproducible solvent blank. Since an accurate aliquot of 2.5 ml of solvent is collected, it is possible to add 2.5 ml of solvent to each of the tubes of the standard curve, compensating for its effect. With 24 duplicate and triplicate plasma samples over a range of 0.1 to 1.5 ng testosterone, the average % difference

$$\frac{\text{larger value - smaller value}}{\text{larger value}} \times 100 \text{ is } 5.2\%, \text{ with a range of } 0.2 \text{ to } 16.5\%$$

In water blanks testosterone was undetectable. An indication of minimum sensitivity is the detection of testosterone at the female level

with a plasma sample of 0.5 ml. From this, and indications from the shape of a "profile" curve, we can obtain by plotting eluate volume vs. ng testosterone equivalent, we can routinely detect 0.5 ng testosterone. We have done the following plasma testosterone determinations:

Subject	Testosterone (ng/100ml)
Functional prepuberal castrate (age 14)	60
Hypogonadism	46
Male climacteric	208

A female patient had a plasma testosterone level of 42 ng/100ml. Subjects receiving testosterone propionate have testosterone levels exceeding 1100 ng/100ml.

Our general agreement with other established methods of plasma testosterone measurement is shown in the following table:

COMPARISON OF PLASMA TESTOSTERONE ASSAY METHODS

REFERENCE	METHOD	NORMAL MALE (ng/100ml T) Mean - SD	Range	# Plasma
Rivarola, M.A. & Migeon, C.J. (36)	Double isotope dilution using acetic anhydride	551 ± 151	373 - 917	7
Bardin, C.W. & Lipsett, M.B. (37)	Double isotope dilution using acetic anhydride	730 ± 260	360 - 1170	24
Gandy, H.M. & Peterson, R.E. (38)	Double isotope dilution using acetic anhydride	670 ± 230	280 - 1140	60
Frick, J. & Kincl, F.A. (39)	Competitive protein binding (TLC x 2)	740 ± 290		21
Maeda, R. et al. (40)	Competitive protein binding (PC)	735 ± 308	273 - 1121	10
Rosenfield, R.L. et al. (41)	Competitive protein binding (TLC)	533 ± 259	238 - 1001	11
Mayes, D. & Nugent, C.A. (35)	Competitive protein binding (PC, TLC, Al_2O_3)	680 ± 180	450 - 960	16
Furuyama, S. et al. (42)	Radioimmunoassay	590 ± 149	365 - 815	13
This laboratory	Competitive protein binding (Sephadex LH-20)	515 ± 164	212 - 896	36

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5. Summary of Specific Highlights of Work to Date

a. Cytological

i) Development of a method for the quantitation of germinal cells.

ii) Development of a method for the quantitation of Leydig cells.

iii) Confirmation of the timing of spermatogenesis in normal men as reported by Heller and Clermont [11], with X-ray as the tool [16].

iv) An outline of the quantitative response of the various germinal cell types to irradiation at dose levels of approximately 10r to 600r.

v) Classification of the dose-response for the various germinal cell types.

Low doses (10r - 100r) - spermatogonia affected

Intermediate doses (100r - 300r)- spermatogonia affected as well as spermatocytes (but the latter do not appear visibly damaged under the light microscope)

High doses (400r - 600r)- all cell types are affected; spermatids, however, are not visibly damaged using the light microscope.

vi) Evaluation of the effect of biopsy upon sperm count.

vii) Ultrastructural description of four types of human spermatogonia, a first in the field of electron microscopy [8].

viii) Preliminary identification of another type of spermatogonium, perhaps radiation-resistant, from data on light microscopic examination of X-ray-depleted biopsies.

ix) Human germinal cells embedded in Epon and viewed under the light microscope have been described for the first time.

x) Determination of ductular transport time of mature spermatozoa from the time they are released from the Sertoli cell cytoplasm until they appear in the ejaculate (6).

xi) Discovery that the morphology of sperm during the recovery is normal, that is, the testis cleans itself of abnormal cells.

xii) Quantitative inspection of the recovery of all doses studied following irradiation. The earliest spermatogonial recovery begins at approximately 150 days for all doses.

xiii) Individuals given the same dose of X-ray irradiation respond in a slightly different manner, i.e., recovery may take longer in one than another.

xiv) Discovery that humans are unique with regard to germinal epithelium recovery, as compared to other mammals studied. Surviving human spermatogonia do not repopulate before differentiation occurs as with mouse, rat, etc. In humans the spermatogonia differentiate rapidly into more mature cells. Thus during the process of depletion in mouse (21), spermatogonia surviving irradiation damage quickly renew

themselves and repopulate the seminiferous tubules. Only after such renewal takes place does differentiation occur. In man, following doses of 15 to 50r, the surviving spermatogonia do not repopulate the entire seminiferous tubule, but after some slight renewal effort, quickly differentiate. This further denudes the germinal epithelium and further lowers sperm count. Later, as more spermatogonia begin renewal but also begin differentiation, this phenomenon results in a great delay in recovery at all doses (15 to 600r).

xv) Determination of the duration of each cell type by evaluation of germinal cell quantitation [5].

xvi) Immediate effect of X-ray on sperm morphology. During depletion sperm remain normal following irradiation at doses below 400r; at 400r and above, sperm morphology is severely damaged in the first 67 days after X-ray, indicating damage to cells that were spermatids at the time of irradiation.

b. Hormonal

i) Accumulation of further evidence toward substantiating the "utilization hypothesis" [43], that is, that gonadotropins are directly related to the functional status of the testes. This assumes that the germinal elements in the testes normally utilize gonadotropin and that following cellular depletion of the tubule less gonadotropin is utilized. This results in more gonadotropin appearing in the venous effluent of the testes, the general circulation and in the urine. This is consistent with our finding that total gonadotropins are increased as the germinal epithelium is denuded following

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irradiation.

ii) Data showing that urinary and plasma follicle-stimulating hormone increases as the germinal epithelium is depleted following irradiation, and decreases as repopulation occurs. Also, data revealing that urinary ICSH does not increase following irradiation.

iii) Adaptation of a method for the radioimmunological determination of plasma ICSH, which shows a rise in plasma ICSH following irradiation.

iv) Leydig cell function appears to be depressed by higher doses of irradiation as reflected by lowered urinary testosterone levels. Compensatory mechanism seem to be elicited as reflected by increase in plasma ICSH and increase in Leydig cell numbers at high dose levels.

v) Administration of exogenous human chorionic gonadotropins following irradiation reveals that the Leydig cells are as capable of responding to this stimulus as are normal Leydig cells. This may explain their response to the elevated endogenous ICSH.

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