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The Effects of Ionizing Radiation on Endocrine Cells VII. Androgen Synthesis and Metabolism by Rat Testicular Minced and Teased-Tubular Preparations After 450 r of Whole-Body X-Irradiation.

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Running Head: X-IRRADIATION AND ANDROGEN METABOLISM

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ABSTRACT

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Male rats (Holtzman strain) were irradiated when 12 weeks of age and sacrificed at 3-day intervals. Minced preparations of testicular tissue from irradiated and control rats were incubated with pregnenolone-7α-H^3 and progesterone-4-C^{14} for androgen synthesis studies. Minced preparations were also incubated with testosterone-4-C^{14} to measure rates of inactivation of this androgen. Teased-tubular preparations from both groups of animals were incubated with pregnenolone-7α-H^3 and progesterone-4-C^{14}. Short-term cyclic variations were seen in androgen synthesis by both the minced and the teased-tubular preparations for control as well as irradiated animals. An increase in androgen synthesis on day-1 corresponded with previously reported decreases in melatonin synthesis by pineal glands. Testosterone synthesis from irradiated rats was diminished after day-1 by both the minced and teased-tubular preparations. Cyclic changes in testosterone synthesis with time were observed for both the control and irradiated animals, but the irradiated animals failed to respond quantitatively to the cyclic increase in androgen synthesis when compared with the control animals. Testosterone inactivation was increased by irradiation, but less androstenedione was formed from testosterone after irradiation as a result of irradiation suggesting that other high-polar metabolites were formed after irradiation.

Rat Testes Irradiation Teased-Tubules Androgens

INTRODUCTION

Steroid biotransformations by adrenal tissue were the first such phenomena shown to be altered by ionizing irradiations *in vitro* (1,2) and *in vivo* (3). Steroid biotransformations by testicular tissue are known to be affected by whole-body irradiation (4), localized irradiation of the testes (5-7), localized irradiation of the head (8), direct irradiation of testicular tissue *in vitro* (9), and by internally deposited radionuclides (10). In addition, other workers have presented evidence for disturbances in androgen synthesis following irradiation (11-13). In this respect, Gunn *et al.* (13), have shown that prostatic weights and Zn^{65} uptake by prostatic tissue are altered in a triphasic manner after either whole-body irradiation (13) or localized microwave irradiation of the testes (14) demonstrating that there are triphasic changes in irradiation (4).

In spite of the above investigations, no attempt has been made to measure the rate of inactivation of testosterone by testicular tissue after irradiation. Even though teased-tubular preparations from rat testes can biotransform progesterone into androgens (15), no attempt has been made to ascertain if the maturation depletion of germinal elements that occurs after irradiation (for recent review see ref. 16) has any effect on androgen synthesis by the seminiferous tubules. This investigation was undertaken to evaluate the ability of the testes to inactivate testosterone, to relate this to the overall ability of the testis to

synthesize androgens after irradiation, and to ascertain what relationship exists between teased-tubular biosynthesis of androgens and histological changes in the germinal epithelium.

MATERIALS AND METHODS

Irradiation and housing of animals

Thirty-five male rats (Holtzman strain) were irradiated with 450 r of whole-body x-irradiation when 12 weeks of age. Thirty-five comparable animals were sham irradiated to serve as control animals. A 250 kvp (Westinghouse Quadrocondex) medical x-ray machine was used to treat the animals. A 1 mm Al and ½ mm Cu filter was used to remove the soft x-rays from the beam. A thimble-type dosimeter (Victoreen Inst. Co.) was used to calculate the dose. A 70 cm distance from mid-plane of rats to focal point was used. The rats were irradiated four at a time in a plexiglass contained that was slowly rotated under the x-ray tube. The average dose-rate was calculated to be 50.2 r/min. in air.

Prior to and after irradiation the animals were housed five per cage in a commercial animal care unit in the small animal laboratory. The room had a southern exposure. The temperature was maintained at 72° F; the relative humidity was maintained at 35%. Laboratory chow and water were given ad libitum. The animals were acclimated to the conditions of the laboratory for one week prior to being treated. Three animals were sacrificed from both the control and the irradiated groups according to the following schedule: 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, and 32 days after treatment.

Preparation of samples for incubation, hematocrits and histological studies

At the designated time intervals, the animals were sacrificed with an overdose of ether anesthesia. The animals were quickly weighed and their testes removed, chilled in ice-cold 0.1 molar phosphate buffer (NaCl, 0.742 g; KCl, 0.038 g; $MgSO_4$, 0.032 g; K_2HPO_4, 1.217 g; $NaHPO_4$ H_2O, 0.173 g per 100 ml of distilled water, pH 7.4), trimmed, weighed, and decapsulated.

Hematocrits were obtained from blood taken by intracardiac puncture using a 1 ml syringe. The blood was transferred to capillary tubes that were plugged and spun in a clinical centrifuge (International Equipment Co. No. y4360) for 60 minutes. Hematocrits were run in duplicate for each animal.

Part of one testis was fixed in buffered formalin and was later imbedded, sectioned, and stained with hematoxylin-periodic acid-Schiffs stain. Approximately one-half of the remaining tissue was minced, and one 0.3 gram aliquot of the mince was taken for incubation with pregnenolone-7α-H^3 and progesterone-4-C^{14}. A second 0.3 gram aliquot was incubated with testosterone-C^{14}. The remaining tissue was placed in a Petri dish that was embedded in a larger bowl of crushed ice. The tissue was covered with the phosphate buffer. A black piece of cloth between the Petri dish and the ice increased the visual contrast between the tissue and background of ice. A large magnifying glass was mounted on a ring stand above the preparation and desk lamps were used for additional lighting. The seminiferous tubules were teased from the testicular mass with fine forceps and were transferred to a 10 ml beaker containing 5 ml of the phosphate buffer that was also imbedded in crushed ice. Approximately 20 minutes were required to tease

enough tubules (0.3 g) for the incubation. At this time, the teased-tubules were harvested from the buffer with the aid of a fine pair of forceps. The tubules were blotted on filter paper (Whatman #1) and were weighed in five ml plastic disposable beakers.

Incubation of samples

Prior to use, the radioactively labeled steroids (New England Nuclear Co.) were purified with paper chromatography. Aliquots of the purified steroids were taken for recrystallization to a constant specific activity with carrier steroid (Sigma Chemical Co.), as described elsewhere (4,17), to insure that the precursor steroids were pure. Pregnenolone-7α-H^3 and progesterone-4-C^{14} were made equimolar by adding carrier steroid to the purified steroids to give a stock solution containing 0.5μc of pregnenolone-7α-H^3 (2.275 mμmoles), 0.05 μc of progesterone-4-C^{14} (2.275 mμmoles), and 20λ of propylene glycol per 2 ml of methanol. Testosterone-4-C^{14} was purified and used at the specific activity specified by the supplier. A stock solution was made up of this steroid containing 0.05μc (0.5 mμmoles) and 10λ of propylene glycol per ml of solution.

At the time of incubation, 2 ml of the pregnenolone-7α-H^3 and progesterone-4-C^{14} stock solution were transferred into two Erlenmeyer flasks (125 ml) and the methanol was blown off with a dry steam of nitrogen gas at 45° C. Similarly, 1 ml of the testosterone-4-C^{14} stock solution was transferred to a third Erlenmeyer flask, and the methanol was evaporated with nitrogen gas. An aliquot of minced tissue, in 10 ml of the phosphate buffer, was transferred into one of the flasks containing pregnenolone-7α-H^3 and progesterone-4-C^{14} and was incubated for 30 minutes at 37.5° C in air. Similarly, the aliquot of teased-tubular tissue was transferred in 10 ml of phosphate buffer into the second flask containing

the pregnenolone-7α-H^3 and progesterone-4-C^{14}. The samples were incubated for 2½ hours at 37.5°C in air. A second aliquot of minced tissue in 10 ml of phoshate buffer was transferred to the flask containing testosterone-4-C^{14} and was incubated at 37.5°C in air for 2½ hours.

Termination of incubations and the isolation, identification, and quantification of steroid intermediates

The incubations were terminated by adding an equal volume of acetone. The resulting mixture was heated to the boiling point with constant agitation to precipitate the proteins. The samples were stoppered and kept in the freezer at -20°C until they were analyzed further. At this time the samples were reheated and quickly filtered through Shark Skin filter paper (Schleicher and Schuell Co.). The residue in each flask was exhaustively extracted an additional three times with hot acetone. The acetone from the extracts was combined in 250-ml round-bottomed flasks and evaporated in vacuo with a flash evaporator at 45°C. Steroid metabolites were separated and characterized according to the carrier method of isotope dilution by using paper chromatography and derivative formation (4,17,18). Purity of the isolated and characterized steroids was checked by recrystallizing pooled aliquots of the steroid fractions to a constant specific activity with approximately 35 mg of carrier steroid. The following systems of Zafforoni (19) were used in this investigation: system I, hexane:carbitol; system II, hexane:formamide; and system III, hexane-benzene:formamide. Steroidal derivatives were formed by acetylation with acetic anydride and pryidine (1:1) overnight at room temperature or by mild oxidation with dilute chromic acid in glacial acetic acid (4,17,18).

Carrier steroids were visualized on the chromatograms with an ultraviolet scanner (230mμ wavelength). Radioactively labeled steroid metabolites were visualized with a 4π electronic autoscanner (Nuclear Chicago Actigraph III).

Quantification of steroid intermediates and statistical evaluation of the data

After isolation and characterization of the steroid intermediates, the individual fractions were transferred to liquid scintillation vials with $CHCl_3$:methanol (1:1). The solvent was evaporated and 20 ml of the liquid scintillation cocktail was added to each of the vials. The samples were counted in a liquid scintillation spectrometer (Packard Instr. Co.) utilizing the method of simultaneous equations for dual isotope labeling (20). The equations were evaluated with a digital computer, and the results were expressed as mμmoles of the precursor steroids converted into the steroid metabolites isolated. Nonincubated samples were carried through the extraction and isolation procedures to correct for losses during the identification and characterization procedures.

A paired observation for the comparison of sample means (21) was used to ascertain if these differences were due to treatment with respect to time. A digital computer was used to fit the data to mathematical equations.

RESULTS

Histological findings

At one day post irradiation, there were no significant changes observed in the seminiferous tubules. Four days after treatment all cell types were still present, but there was a definite decrease in the number of mitotic figures. The primary spermatocytes appeared to be slightly swollen. At 7 days post irradiation, there was a definite decrease in

the number of primary spermatocytes. The nuclei of the secondary spermatocytes were very swollen. No mitotic activity was observed. At 10 days post irradiation, there was a paucity of primary spermatocytes. Many of the secondary spermatocytes still had very large swollen nuclei and showed marked degeneration. Debris from the necrotic cells had accumulated in the lumen of the tubules. Mature sperm and spermatids were present in the tubular lumina and adjacent to the layer of secondary spermatocytes. At 13 days post irradiation, virtually no primary spermatocytes were observed. Very few normal secondary spermatocytes were present. Those whose morphology was still distinguishable were markedly swollen. There were large amounts of cellular debris, both adjacent to the degenerating secondary spermatocytes and in the tubular lumina. At 16 days post irradiation, a few primary spermatocytes were seen adjacent to the tubular membrane. However, very little mitotic activity was still observed. There were virtually no secondary spermatocytes present at this time. Some mature sperm lay in the area adjacent to remnants of sustentacular cells. The tubules were extremely devoid of normal appearing cellular elements. At 22 days, primary spermatocytes were seen again adjacent to the tubular membrane. Macrophages were present in the tubular lumina and appeared to be phagocytizing cellular debris. There were virtually no secondary spermatocytes present. Spermatids and mature sperm were also scarce. Much of the tubular lumina at this stage was filled with a very pale pink fibrillar material. Some mitotic activity was observed in the primary spermatocytes although this was not very marked at this time. At 25 days, the number of primary spermatocytes was now increasing at a fairly rapid rate. Very few mature sperm were seen. Many tubules still contained a large amount of

cellular debris near their center. The fibrillar material which had been previously described was very evident in these sections. At 28 days, there was still an extreme paucity of cells within the tubular lumina. Some primary spermatocytes were identifiable, but mitotic activity towards formation of normal sperm was still not evident. There were no significant changes noted at 32 days that were not observed at 28 days. There was still an extreme degeneration of the cellular components of the seminiferous tubules at this latter time interval.

General effects of radiation

A decrease in testicular weight was evident as early as one day after treatment, but maximum loss of testicular weight occurred 10 days post-irradiation (Figure 1). Body weight gains and changes in hematocrits are shown in Figure 2. Body weights changed little except for the first four days after treatment. With the exception of day-25, body weights for the treated animals averaged slightly less than those of the control animals throughout the observation period. The differences in body weight between the control and irradiated animals on days 1, 4, 7, 10, 13, 16, 19, 22, 28, and 32 after treatment was significant ($P < 0.05$).

The diminished hematocrit values (Fig. 2) noted from day-4 through day-32 ($P < 0.001$) were assumed to be due to the irradiation of the animals.

Irradiation effects on steroid biotransformations by testicular minced Preparations

The biotransformation of pregnenolone-7α-H^3 into progesterone and the residual amounts of progesterone-4-C^{14} and -7α-H^3 obtained from incubations of minced testes are illustrated in Figure 3. Statistical analyses of the data show that, from day-13 through day-32, less progesterone remained in the progesterone pool from incubations of tissue

from the irradiated animals than was observed for similarly obtained values for the control animals ($P < 0.05$). Similarly, there was less 17α-hydroxyprogesterone remaining in this steroid pool after incubation of testicular tissue from the irradiated animals than was observed for that from control animals (Fig. 4) from day-22 through day-32 ($P < 0.001$). The androstenedione pool (Fig. 5) was diminished on days 1, 13, 16, and 25 for the treated animals when compared with values for the control animals ($P < 0.05$), but was increased on days 4, 7, 19, 22, and 32 ($P < 0.0025$).

Testosterone isolated from incubation of testicular tissue from both groups of animals (Fig. 6) was consistently less for the irradiated groups than was observed for the control animals from day-4 through day-32 ($P < 0.001$). Quite noticeable was the increase in testosterone for both groups during the period day-16 through day-32. When testosterone-4-C^{14} was incubated with minced testicular tissue, the amount of testosterone recovered from the incubation was diminished from day-13 through day-32 for the irradiated group of animals (Fig. 7). The divergence was greatest on day-32 with no signs of reversal ($P < 0.001$). No significant difference was observed for the data from day-1 through day-13. These data show that testosterone metabolism was increased from day-16 through day-32. Similarly, the amount of androstenedione recovered from these incubations was diminished from day-13 through day-32 with the greatest divergence existing on day-32 (Fig. 8). Thus, the increase in testosterone metabolism was not due to an increased conversion of testosterone into androstenedione.

The biotransformation of steroids by teased-tubular preparations

A histological section of teased-tubular stuffed into an enverted testicular capsule (Fig. 9) show little interstitial cell contamination

and good integrity of the germinal epithelium. The amount of progesterone recovered from incubations of teased-tubular preparations with pregnenolone-7α-H^3 and progesterone-4-C^{14} was not significantly altered by treatment of the animals with x-irradiation from day-1 to day-7 (Fig. 10). It was decreased, however, from day-10 through day-32 ($P < 0.001$). There was a significant difference in the amount of 17α-hydroxyprogesterone that was isolated from each group on day-1 ($P < 0.05$, Fig. 11), but there was no difference between the two groups of animals for this steroid fraction on days 4, 7, 10, 13, 16, 19, and 32.

Androstenedione was significantly increased in samples from treated animals compared to those from control animals on days 1, 4, 10, and 13 ($P < 0.05$, Fig. 12). It was also reduced in samples taken on days 7, 16, 19, 22, 25, 28, and 32.

Testosterone recovered from the above incubations (Fig. 13) was decreased from day-4 through day-32 ($P < 0.05$). Of interest is the fact that there was a marked increase in testosterone in samples from both groups of animals from day-19 through day-25. During this period, the difference between the control and treated groups was greater numerically than it was from day-7 to day-16 or from day-28 to day-32. A similar observation was made for testosterone recovered from incubations with minced tissue (Fig. 7), except that the difference was more marked in the teased-tubular incubations. When the control and irradiated values for testosterone were fit to a mathematical expression (Figs. 14 and 15), the data closely fitted the mathematical expression $y = ab^{x_1} c^{x_2} d^{x_2^2} e^{x_2^3}$ ($R^2 = 0.904$ and 0.875, respectively).

DISCUSSION

Our data show that teased-tubules as well as minced testicular tissue synthesize androgens. When, however, incubation time is taken into account,

the minced tissue produced more androgens than did the teased-tubular preparations. This observation agrees with earlier findings of Christensen and Mason (15), except they did not demonstrate that pregnenolone is a better substrate for testosterone synthesis than progesterone as has been done in this investigation. In this respect, Christensen and Mason (15) were the first to show that teased-tubular preparations could synthesize androstenedione and testosterone from progesterone. Other recent investigations now corroborate this observation and show that teased-tubular preparations can biotransform pregnenolone-7α-H^3(16,22-23) and progesterone-4-C^{14}(16,22-24), but not cholesterol (25) into androgens. Inspection of the data reveals an apparent cyclic variation in testosterone production in both the control and irradiated groups from day-16 through day-32 (Figs. 6 and 13). Noteworthy is the observation that the difference in testosterone synthesis between the control and irradiated groups was greater for both the minced and teased-tubular preparations during the period from day-16 through day-32 than it was from day-4 through day-13. One exception was day-4 for the teased-tubular preparation. Apparently, the testes of the irradiated group cannot respond quantitatively to gonadotropins as do those of the control animals. In studies with human patients (26), an increase in gonadotropins in the urine was observed after irradiation. These data suggest that the hypothalamus and pituitary attempt, through the interstitial cell stimulating hormone, to maintain a normal rate of testosterone production by the testes. Irradiated testes may not be capable of adequately increasing testosterone synthesis on demand, and the biochemical lesion, therefore, becomes more pronounced when the demand for androgen production is increased. In support of this concept, Witschi (27) reported, as early as 1932, that circulating gonadotropins increased in animals following destruction of the

germinal epithelium by x-irradiation. Other workers (13, 28-33) have proven that androgens and gonadotropins have a radioprotective action on the testes after irradiation, suggesting that inadequate amounts of androgens are present in radiation damaged testes to support maintenance and repair processes. Although irradiated testes can respond to exogenous gonadotropins, in most cases the response has been reported as being diminished by irradiation (28-32, 34, 35), supporting the conclusion that the male gonad is incapable of responding quantitatively to gonadotropins after irradiation when compared to normal testes.

Of significance is the fact that much more prenenolone-7α-H^3 was converted into testosterone by the teased-tubular preparations than by the minced preparations. This observation is similar to what has been observed for the conversion of pregnenolone-7α-H^3 into testosterone during the first 15 minutes of incubation for mouse (17) and rat testes (18,36).

An increase in testosterone synthesis by both minced and teased-tubular preparations has also been observed on day-1 in samples from animals subjected to x-irradiation in this investigation. This increase in androgen synthesis occured concomitant with a decrease in melatonin synthesis by the pineal gland 24 hrs after exposure to 450 r (37), and 1 and 24 hrs (38) after 350 r of whole-body x-irradiation. In this respect, melatonin has a direct action on androgen synthesis <u>in vitro</u> (39). Similarly, the increase in melatonin synthesis 24 hrs after irradiation (34) corresponds with the decrease in testosterone synthesis noted in this experiment (Figs. 6 and 13). Although other workers (15) have shown that rat teased-tubular preparations can synthesize androgens, this is the first report of this phenomenon being correlated with physiological (cyclic activity of the controls and irradiated groups) or pathological (irradiation induced) conditions. The fact that

testosterone synthesis by teased-tubular preparations was not markedly altered by the depletion of the germinal epithelium suggests that the biotransforming system for androgens must be associated with a more stable cell-type (either the Sertoli cells or the cells comprising the tubular wall).

As seen in Figure 7, the conversion of testosterone to other metabolites increased after irradiation, while less testosterone was converted into androstenedione. This observation is significant, in that the effective quantity of androgens at the spermatogenic level could be depressed further by an increased inactivation of testosterone. Although other workers have shown that rat testicular tissue can metabolize testosterone to several different metabolites (40), this is one of the first reports of a change in testosterone inactivation associated with a pathological state. The increase in testosterone inactivation, occurring concomitantly with a decrease in germinal elements, suggests that the enzymes for inactivation of androgens do not reside in the germinal cells. This phenomenon is currently being investigated in more detail in our laboratory.

Changes in the hematocrit and in body weight occur after irradiation (Fig. 1) and hematocrit changes up to day-25 paralleled the changes in testosterone synthesis. Body weight changes were maximal during the first four days after irradiation, but were minimal thereafter.

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LEGENDS FOR FIGURES

Fig. 1. Losses in testicular weights in animals irradiated with 450 r of whole body x-irradiation when 12 weeks of age.

Fig. 2 Changes in the body weights and of hematocrits from animals irradiated with 450 r of whole-body irradiation.

Fig. 3 Residual progesterone isolated from testicular minced preparations that had been incubated with pregnenolone-7α-H^3 and progesterone-4-C^{14} after the testes were obtained from animals irradiated with 450 r of whole-body irradiation expressed on a per animal basis.

Fig. 4 17α-Hydroxyprogesterone isolated from testicular minced preparations incubated with pregnenolone-7α-H^3 and progesterone-4-C^{14} after the testes were obtained from animals irradiated with 450 r of whole-body irradiation expressed on a per animal basis.

Fig. 5 Androstenedione isolated from testicular minced preparations from animals irradiated with 450 r of whole-body irradiation and incubated with pregnenolone-7α-H^3 and progesterone-4-C^{14} expressed on a per animal basis.

Fig. 6 Testosterone formed from pregnenolone-7α-H^3 and progesterone-4-C^{14} when testicular minced preparations from rats irradiated with 450 r of whole-body x-irradiation were incubated <u>in vitro</u> expressed on a per animal basis.

Fig. 7 The recovery of testosterone from incubations of minced testicular preparations from control and irradiated animals with testosterone-4-C^{14} after whole-body irradiation with 450 r expressed on a per animal basis.

Fig. 8 The recovery of androstenedione from incubations of minced testicular preparations from control and irradiated animals with testosterone-4-C^{14} after whole-body irradiation with 450 r expressed on a per animal basis.

Fig. 9 Histological appearance of teased-tubules stuffed into an inverted testicular capsule--fixed in buffered formalin, stained with hematoxylin and PAS. Note the absence of interstitial cells in this preparation.

Fig. 10 The recovery of progesterone from incubations of rat teased-tubular preparations with pregnenolone-7α-H^3 and progesterone-4-C^{14} after treatment of animals with 450 r of whole-body x-irradiation expressed on a per animal basis.

Fig. 11 The recovery of 17α-hydroxyprogesterone from incubations of rat teased-tubular preparations with pregnenolone-7α-H^3 and progesterone-4-C^{14} after treatment of animals with 450 r of whole-body x-irradiation expressed on a per animal basis.

Fig. 12 The recovery of androstenedione from incubations of rat teased-tubular preparations with pregnenolone-7α-H^3 and progesterone-4-C^{14} after treatment of animals with 450 r of whole-body x-irradiation expressed on a per animal basis.

Fig. 13 The recovery of testosterone from incubations of rat teased-tubular preparations with pregnenolone-7α-H^3 and progesterone-4-C^{14} after treatment of animals with 450 r of whole-body x-irradiation expressed on a per animal basis.

Fig. 14 Testosterone values taken from Figure 12 for the control group

fit to a mathematical equation ($y = ab^{x_1} c^{x_2} d^{x_2^2} e^{x_2^3}$) with the goodness of fit ($R^2 = 0.904$).

Fig. 15 Testosterone values taken from Figure 12 for the irradiated group fit to a mathematical equation ($y = ab^{x_1} c^{x_2} d^{x_2^2} e^{x_2^3}$) with a goodness of fit ($R^2 = 0.875$).

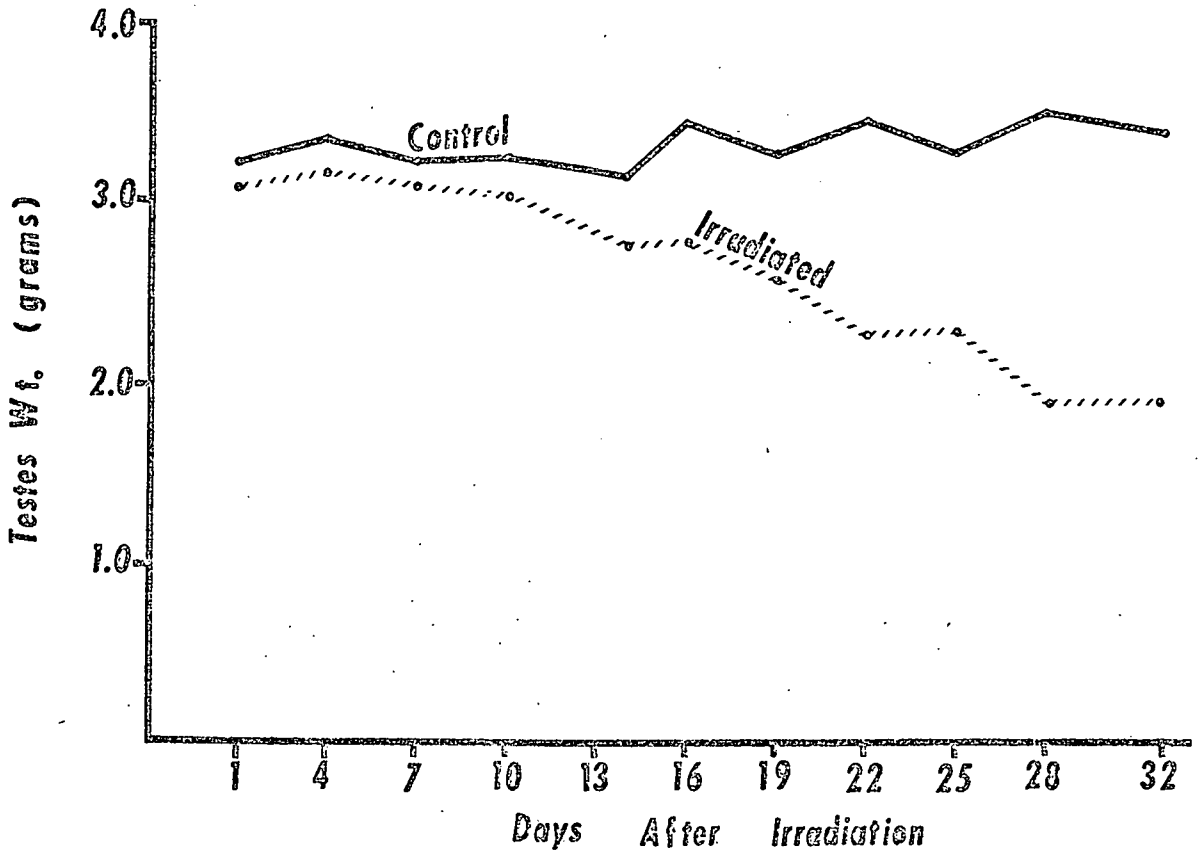

Fig. 1

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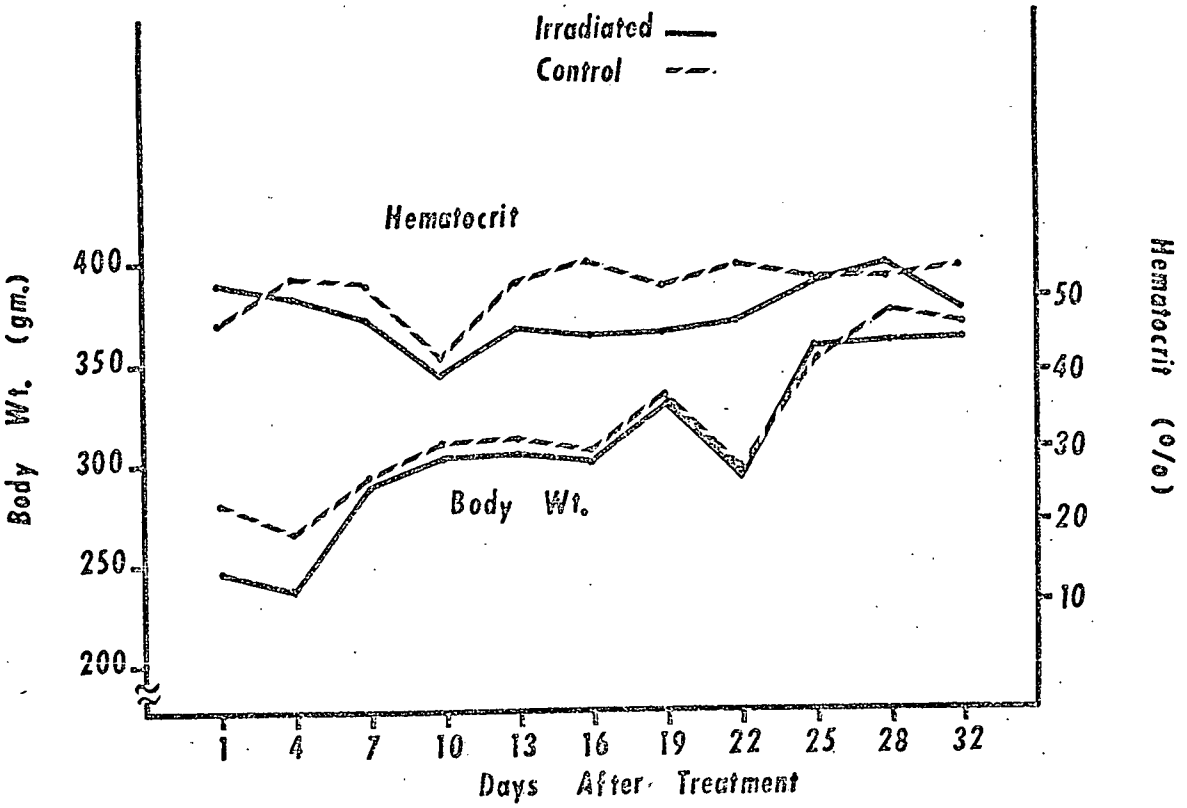

Fig. 2

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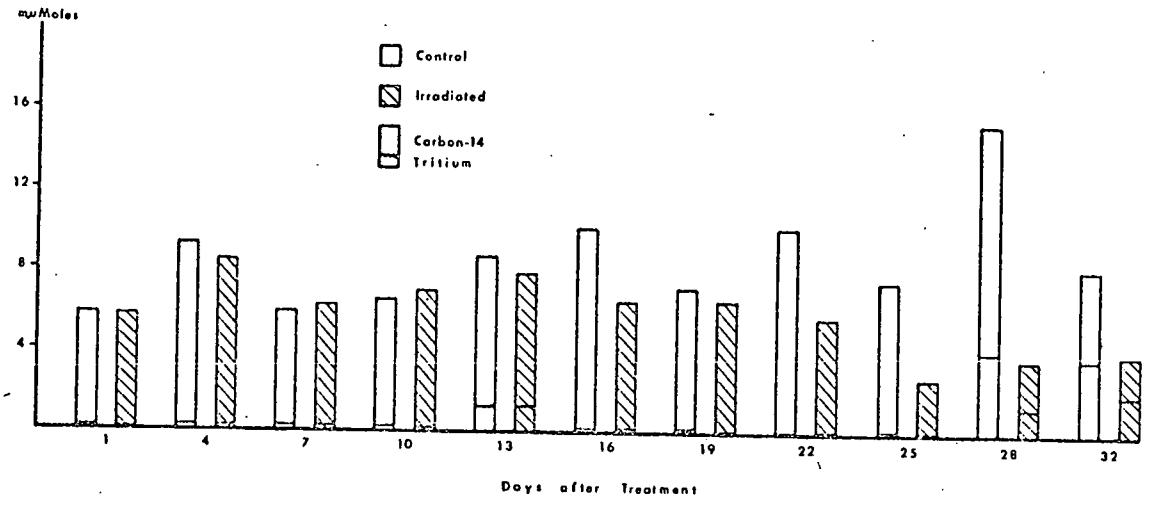

Fig. 3

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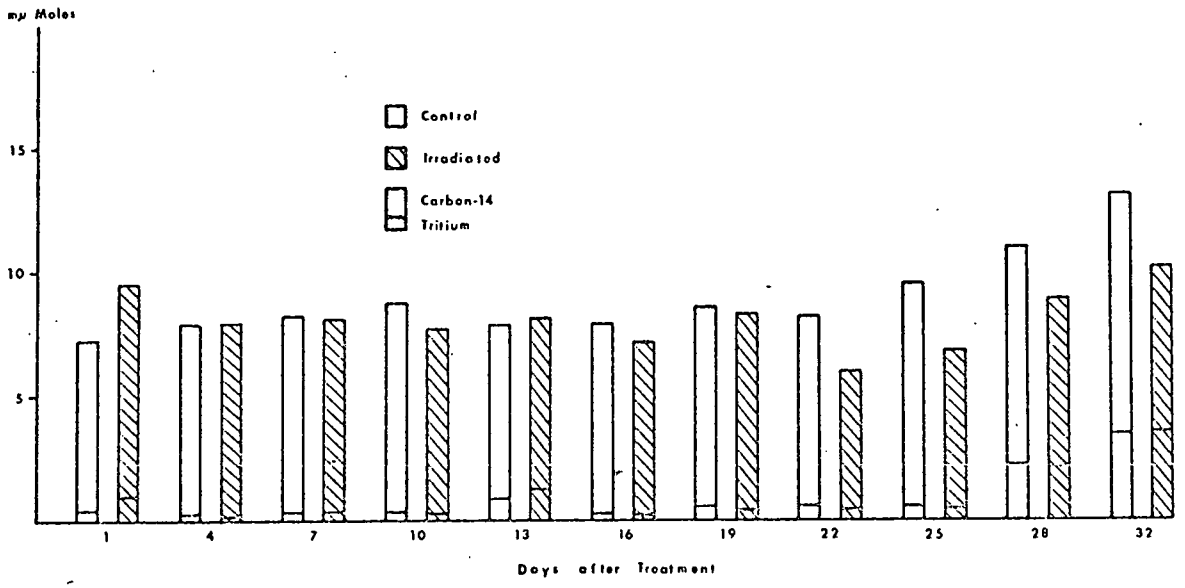

Fig. 4

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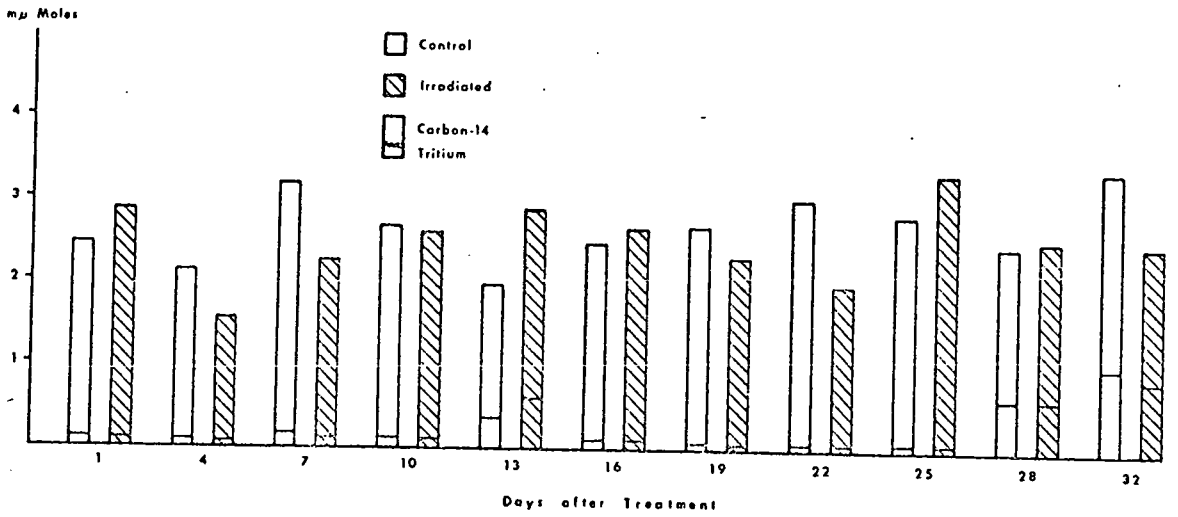

Fig. 5

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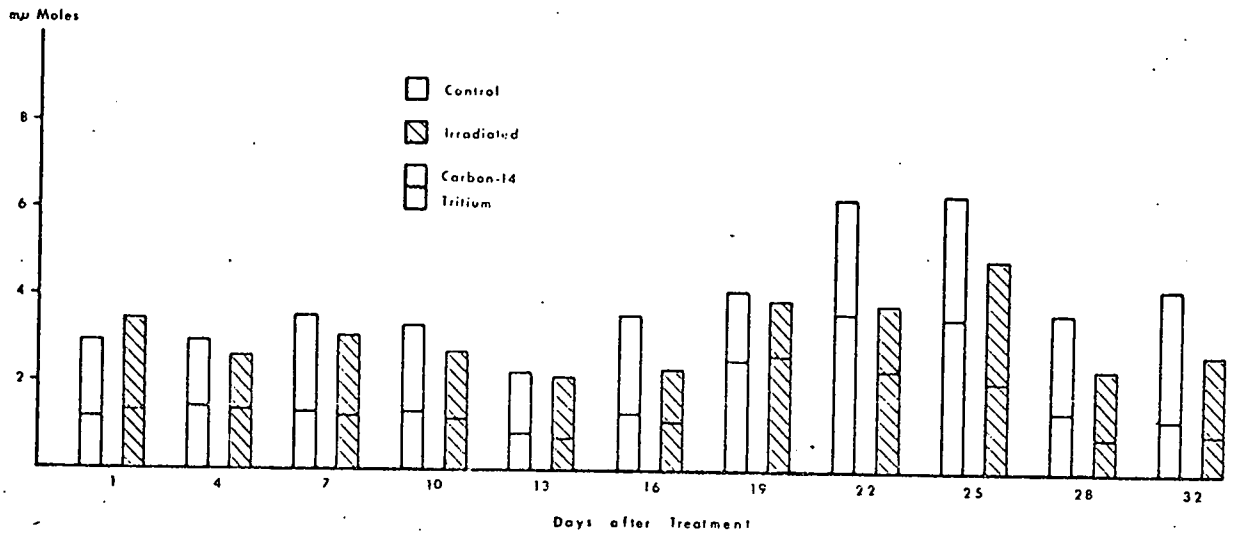

Fig. 6

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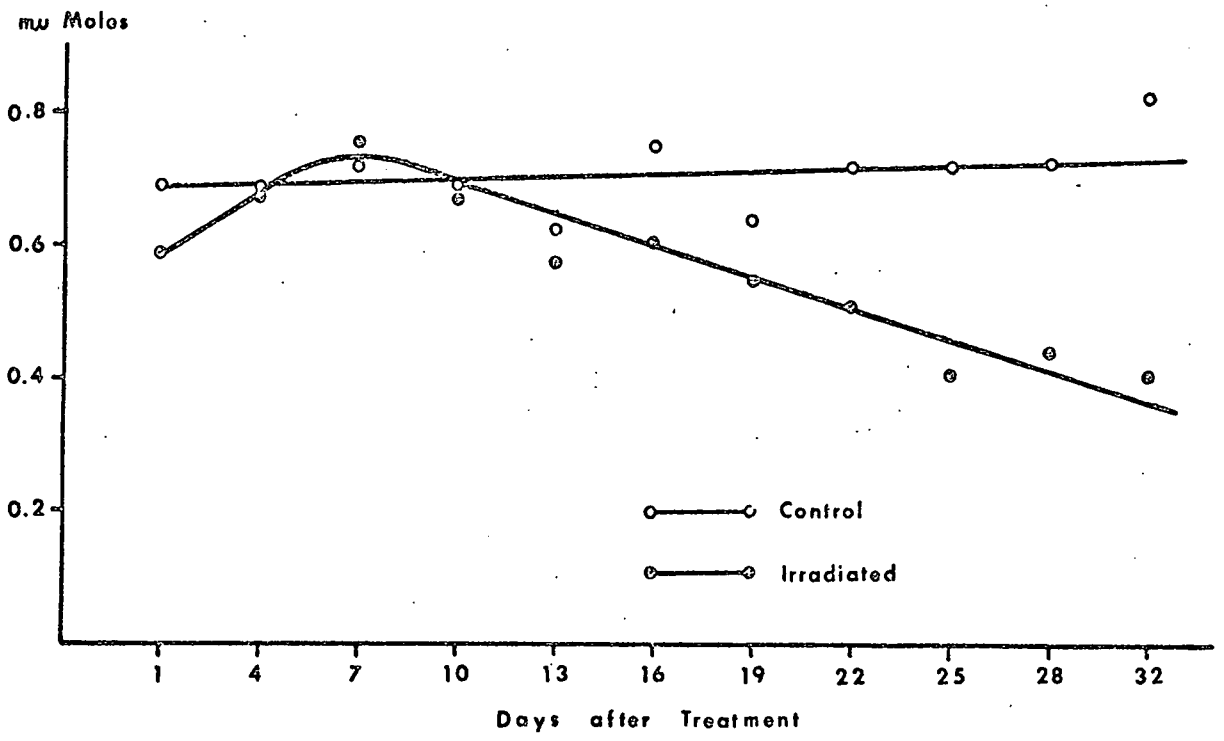

Fig. 7

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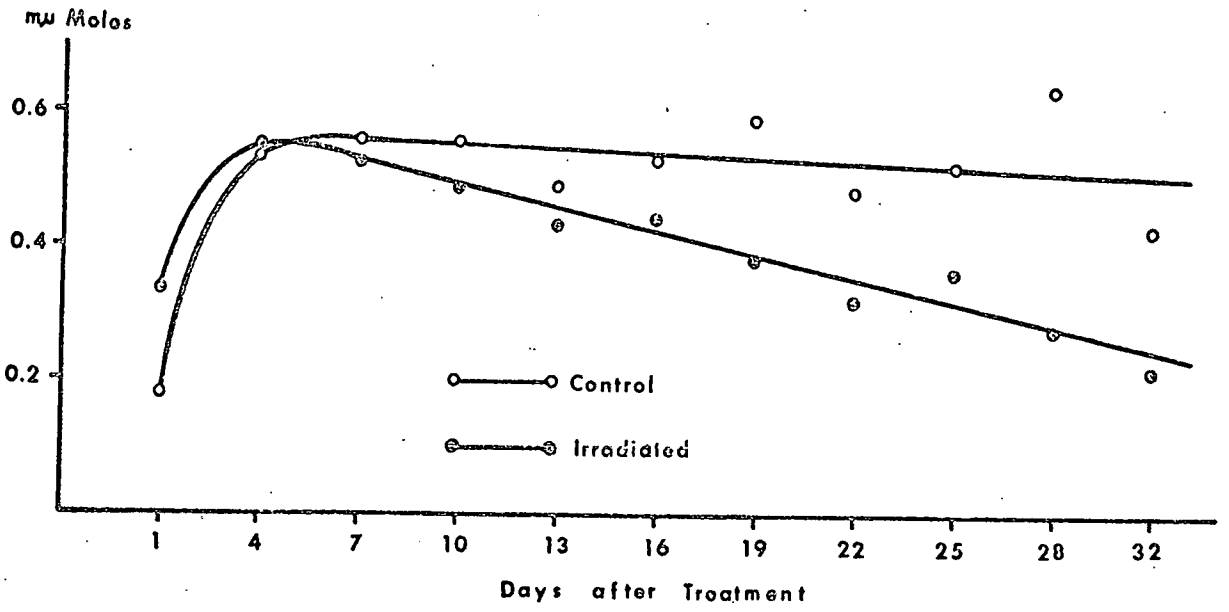

Fig. 8

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Fig. 9

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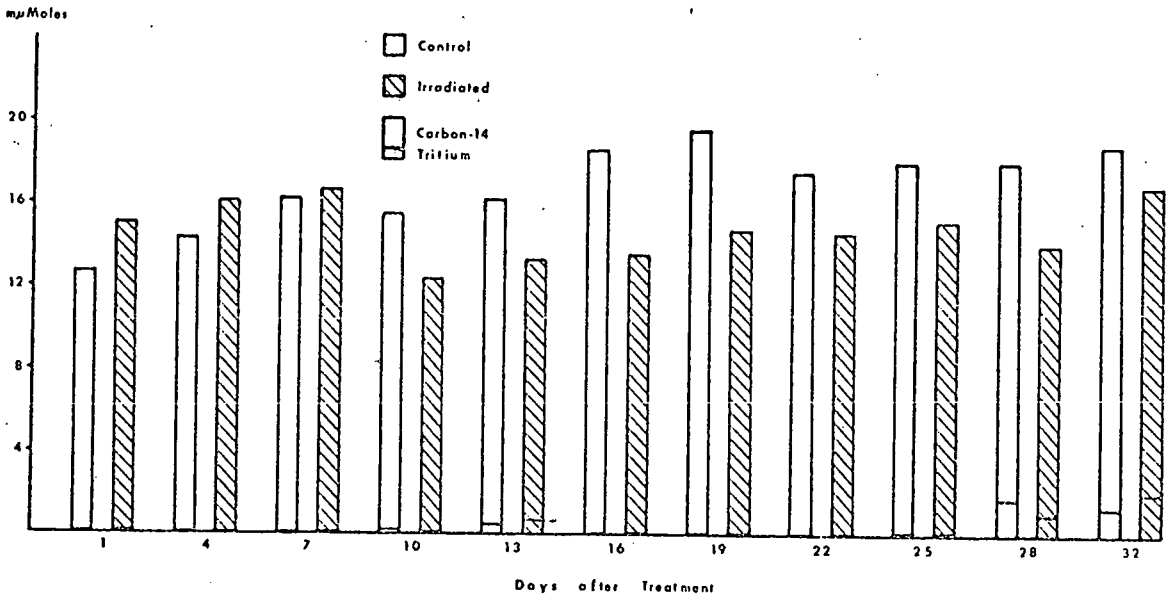

Fig. 10

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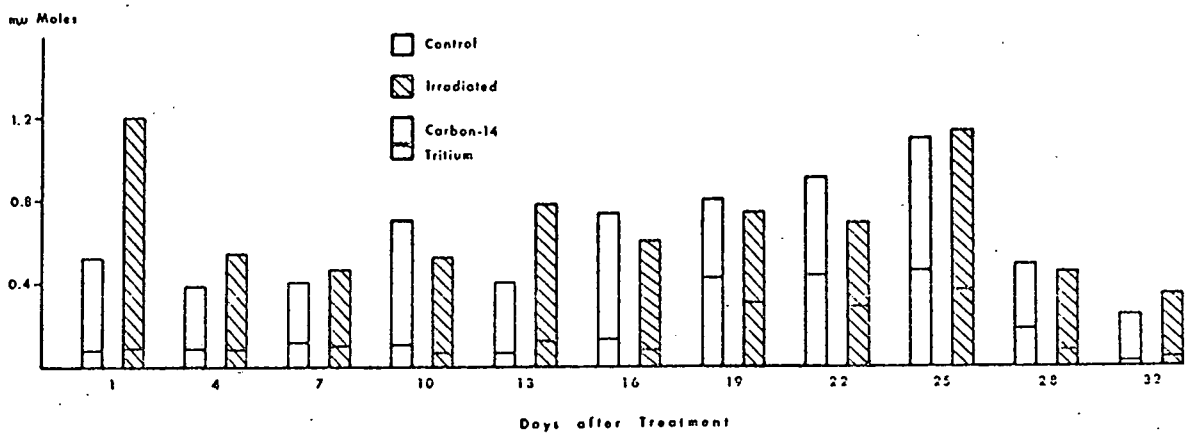

Fig. 11

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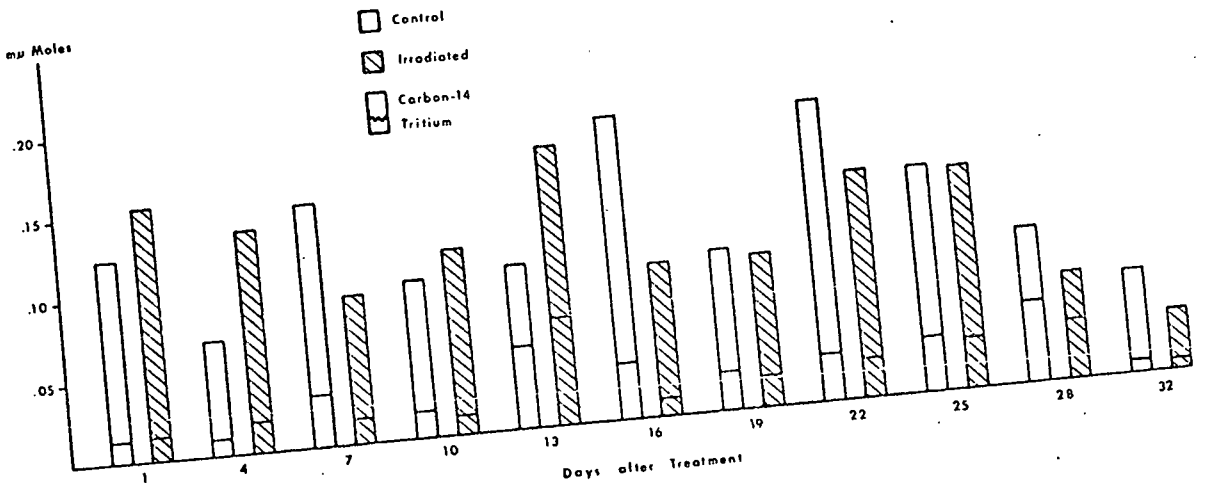

Fig. 12

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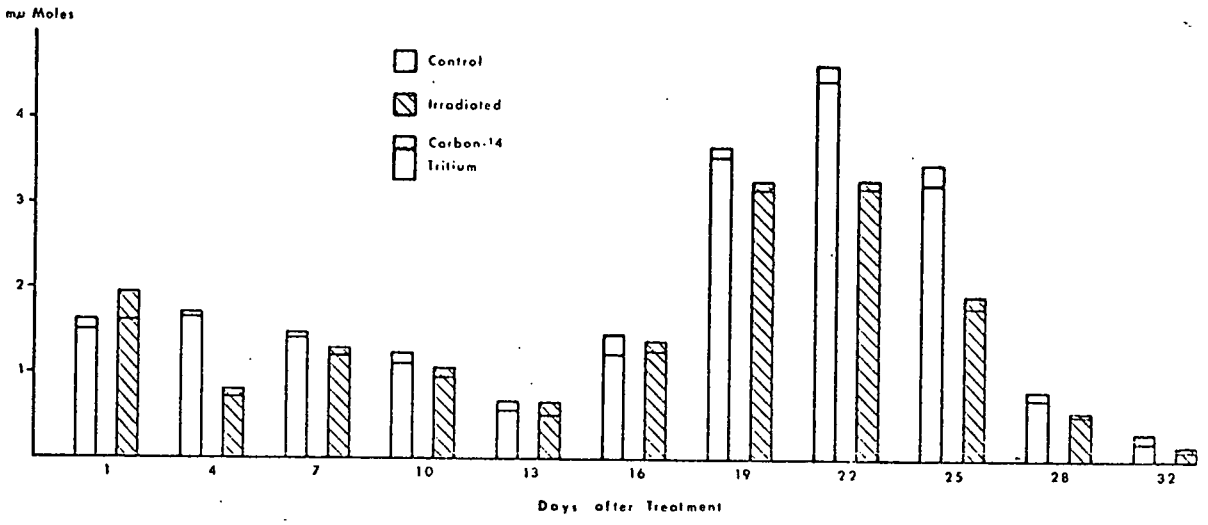

Fig. 13

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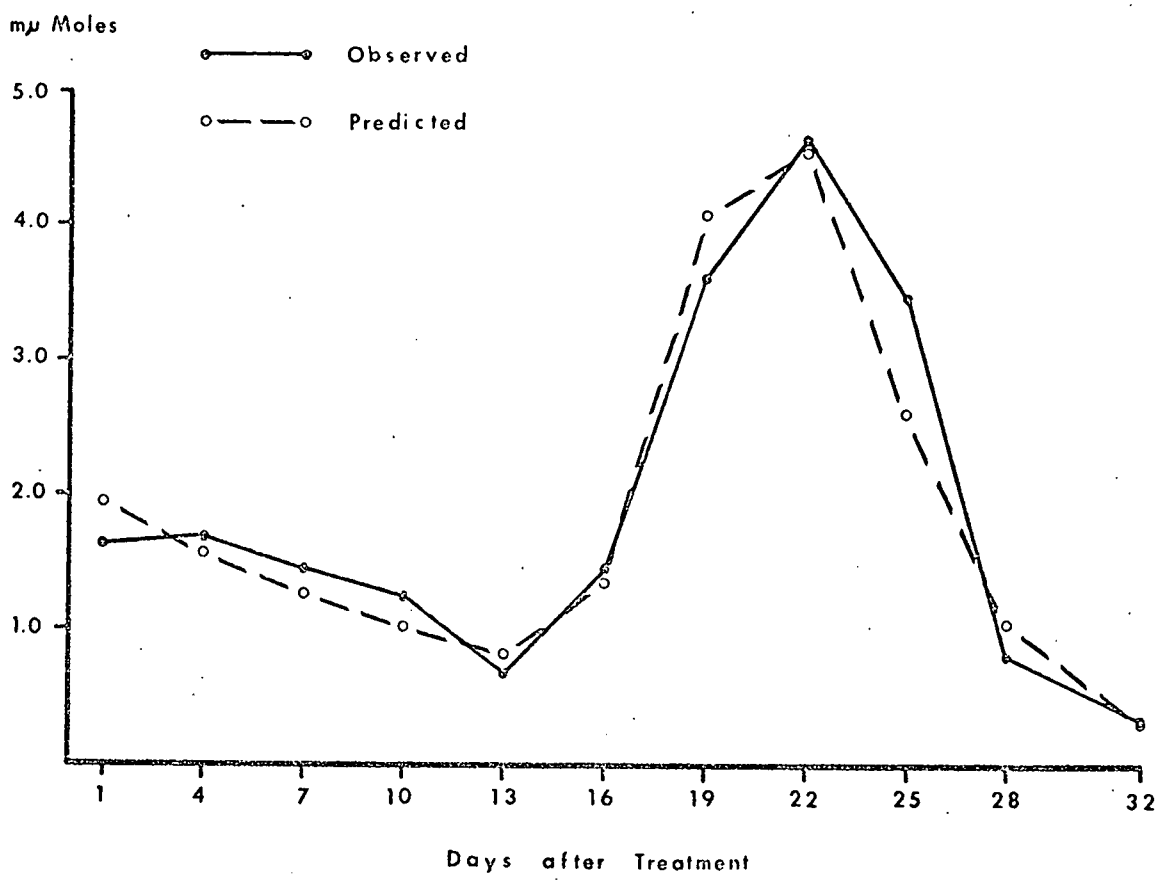

Fig. 14

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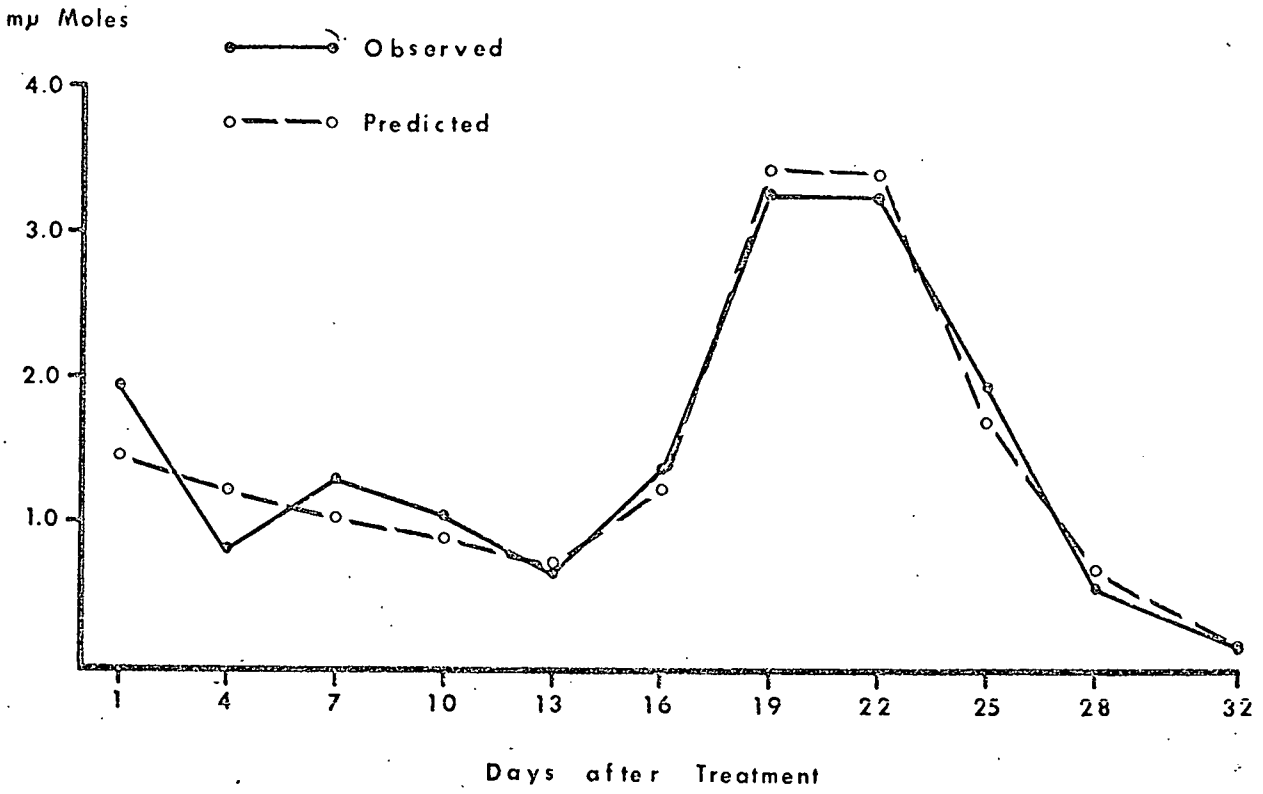

Fig. 15

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