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Cartilage Metabolism during Growth Retardation following Irradiation of the Head of the Neonatal Rat[1] (41534)

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Abstract. The heads of 2-day-old male and female rats were irradiated with a single dose of 600 rads X irradiation, a dose which is known to stunt body weight, tibial length, and tail length, in order to ascertain its effect on synthesis by cartilage of sulfated proteoglycans, DNA, chondroprotein, and collagen as determined by utilization of [^{35}S]sulfate, [Me-^{3}H]thymidine, [1-^{14}C]leucine, and [3,4-^{3}H]proline, respectively. Data have been collected at 20–21, 23, 41–45, and 70–71 days of age. In comparison to controls, growth in body weight, tibial length, and tail length was significantly retarded in irradiated rats of both sexes. Although slow catch-up growth was observed with respect to tail length in both sexes and tibial length in females, a significant deficit in body weight in irradiated rats in both sexes remained at 70–71 days. Cartilage metabolism as evidenced by incorporation of the labeled substances showed no significant disturbance just prior to weaning (20–21 days) or after completion of the principal growth surge (70–71 days). Reduced sulfate and thymidine incorporation attributable to a brief period of undernutrition associated with weaning occurred in head-irradiated rats immediately following weaning (23 days). Increased isotope incorporation occurred at 41–45 days of age in cartilage of irradiated rats incubated with labeled sulfate, leucine, and proline; it did not increase with labeled thymidine. We conclude that neonatal head irradiation slows the rate of growth through the age of most rapid postnatal growth in normal rats. The pattern of cartilage metabolism during this time can be the result either of stimulation by a factor other than somatomedin, or selective inhibition of cartilage thymidine incorporation acting in combination with somatomedin.

Stunting of body weight (1–4) and tail length (3) occurs in rats following head X irradiation during the neonatal period. Previous observations have shown that the growth retardation is related to the dose of radiation, that catch-up growth of body weight and tail length does not occur by 121 days of age, and that normal proportions of body weight and tail length tend to be maintained or restored to normal after weaning even in the absence of catch-up growth (5, 6). Partial head irradiation has shown that bilateral irradiation is required for growth retardation, that irradiation of structures near the midline produces the same effect on growth as whole head irradiation with the same dose, and that direct irradiation of the pituitary is not required for growth retardation (7). The growth response and the neuropathological effects of head irradiation decrease geometrically with age during the first 10 days of life in the rat (8).

The mechanism responsible for remote (abscopal) effects of head irradiation on skeletal growth is unknown. Injections of highly purified bovine growth hormone, L-thyroxine, or both to irradiated rats from 30 through 40 days of age are ineffective in altering the pattern of stunted growth (3). Additionally, pituitary concentrations of bioassayable growth hormone and of thyroid-stimulating hormone in the head irradiated rats at age 23, 42, and 121 days have been normal (9).

The present studies were performed in order to determine whether information on cartilage metabolism in head-irradiated rats undergoing retardation of skeletal growth could provide clues as to the presence or absence of specific stimulators and/or inhibitors of chrondrocyte function. The observed patterns of cartilage metabolism suggest that stimulation and inhibition of different functions of

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cartilage may occur simultaneously. The significance of the findings in regard to control of catch-up and proportionate growth is discussed.

Materials and Methods. *Animals.* One hundred ten Long–Evans rats, 60 males and 50 females, used in this study were bred in our facilities from stock obtained from Simonsen Laboratories, Gilroy, California. The animals were maintained in fresh filtered air, 35 to 70% relative humidity, at 21.1 to 23.3°. Lighting was set for a 14-hr day. Purina Lab Chow and tap water were given *ad lib.* Animal handling and all measurements were carried out by the same individual.

The pregnant rats were kept, one to a cage, in hanging wire mesh cages, until the 14th day of gestation when each was transferred to a box-type cage containing a dustless wood shaving bed. At 2 days postpartum the litter was reduced to eight pups, four of each sex when possible. At weaning, the young rats were transferred to individual cages. Mothers were rested for 14 days before rebreeding and were discarded after the fourth litter. Breeder males were discarded after 18 months of age.

Irradiation technique. All animals were irradiated or sham irradiated while restrained in Teflon holders and placed under a ¼-in.-thick lead shield. The irradiated animals' heads from 2 mm behind the ear pinnae were exposed to the beam. The sham-irradiated rats were placed entirely under the shield. Groups were irradiated by litters with littermates divided into irradiated and sham-irradiated groups. The shield assembly with animals was rotated under the X-ray beam at 16 rpm. The beam consisted of 100 KVP X rays filtered by 2.0-mm Al; the half value layer was 2.3 mm Al, corresponding to a 50% dose at 3.2 cm of tissue. Tube to target distance was 40 cm; field diameter was approximately 25 cm; the animals' heads were 7.5 cm from the field center. The animals' heads rarely exceeded 1 cm in thickness; thus the exit doses were greater than 90% of the entrance doses.

Dose was measured using a Victoreen Condenser-R-Meter Model 570 and 100 R ion chamber, Model 131, as a secondary standard. A Victoreen Radocon II dose rate meter was compared with the standard chamber and subsequently used as a reference exposure rate and total exposure monitor at the field center in most experiments. Lithium fluoride thermoluminescence dosimeters were implanted in the heads of sacrificed 2-day-old rats in order to determine the dose variation at different depths in the brain. These measurements demonstrated that uniformity of dose within the animals' heads was better than ±5%; absolute dose values at the centers of the brains were 1.05 times the values indicated by the Radocon probe at the field center. Each irradiation was carried out at a dose rate of 20 rad/min at the midline of the animals' heads. Based on the integrated dose reading of the Radocon monitor an absorbed dose of 600 rad was delivered to the brains. The dose under the lead shield was undetectable.

Growth measurements. At intervals of a week or less from 20 through 71 days of age, body weight was measured to the nearest 0.1 g and tail length was measured to the nearest 0.1 cm by the technique of de Groot (10). Sacrifices of irradiated and sham-irradiated littermates were carried out at ages 20–21 days, 23 days (2 days postweaning), 41–45 days, and 70–71 days. At sacrifice the right tibia was removed, stripped of soft tissue, and measured for its greatest length to the nearest 0.1 mm with a vernier sliding jaw caliper.

Cartilage incubation. Animals were sacrificed by decapitation between 0900 and 0930 hr. Costal cartilage was prepared for incubation by the method described for bioassay of somatomedin (11). The incubation medium consisted of Krebs-phosphosaline buffer, pH 7.4, containing amino acids and a supplement of glucose, penicillin, and streptomycin as described for the bioassay of somatomedin (11) with the modification that serine was omitted because of its marked stimulatory effect on sulfate uptake by cartilage (11). Cartilage segments from each side of the rib cage were divided between medium containing 10% normal rat serum (NRS) and medium without added NRS. The NRS was from a pool of 90-day-old male Long–Evans rats with a somatomedin potency by bioassay of approximately 1.0 unit/ml. Cartilage from the right side was incubated in 2.0 ml medium containing 1.0 μCi carrier-free [^{35}S]sulfate (New England Nuclear Corp., Boston, Mass.) and 2.0 μCi [Me-^{3}H]thymidine (ICN, Irvine, Calif.; sp act 55 Ci/mmole). Cartilages from the left

side were incubated in 2.0 ml medium containing 0.375 μCi L-[1-^{14}C]leucine (ICN; sp act 40 mCi/mmole) and 1.0 μCi L-[3,4-^{3}H]proline (ICN; sp act 56 Ci/mmole). After 21 hr incubation the medium was decanted from all cartilages. Boiling water was poured into each tube with cartilages and allowed to stand for 15 min, then decanted. Tubes containing cartilages with labeled sulfate and thymidine received 2 ml supersaturated Na_2SO_4 and then were incubated for 3 hr at 37.5°. At the end of 3 hr incubation the Na_2SO_4 was decanted, and the cartilages were washed three times with tap water. Cartilages incubated with labeled leucine and proline were washed with seven changes of tap water over a 3-hr period. The wet cartilages were blotted, weighed, and hydrolyzed at 100° in 96% formic acid. Radioactivity in duplicate aliquots was determined by liquid scintillation counting. Simultaneous assay of ^{3}H and ^{35}S or of ^{3}H and ^{14}C concentrations in the samples was performed by the channels-ratio method.

Statistical calculations. Significance of data was determined by a one-tailed *t* test.

Results. *Physical growth.* Table I contains body weight, tail length, and tibia length data. Body weights of irradiated males and females were less than those of respective controls at each age studied. Tail lengths of irradiated animals were less at 21 days and at 41–45 days in both sexes and in males at 23 days. There was no difference in tail length in either sex at 70–71 days. Tibial lengths were less in irradiated rats of both sexes at 20–21, 23, 41–45 days; at 70–71 days only irradiated males showed significant tibial shortening.

Cartilage metabolism. Incubation with [^{35}S]sulfate (Fig. 1a). At 20–21 days ^{35}S incorporation by cartilage in medium without NRS did not differ significantly between irradiated and control rats of either sex. The addition of NRS produced a slight increase over control values which was significant only in males. At 23 days irradiated rats of both sexes showed a significant decrease in cartilage uptake of ^{35}S in both media. At 41–45 days the pattern resembled that of 20–21 days but with greater uptake in irradiated rats, significant only in males. There was a slight decrease in uptake of ^{35}S in cartilage of both sexes at 70–71 days significant only in males and only in medium with NRS.

TABLE I. BODY WEIGHT, TAIL LENGTH, AND TIBIA LENGTH AT DIFFERENT AGES OF MALE AND FEMALE RATS FOLLOWING X IRRADIATION OF THE HEAD ONLY AT 2 DAYS OF AGE AND OF SHAM-IRRADIATED CONTROLS

	Age (days)	Males			Females		
		Irradiated	Controls	*P*	Irradiated	Controls	*P*
Body weight (g)	20–21	35.0 ± 2.0 (9)	49.9 ± 1.5 (7)	<0.005	35.9 ± 2.1 (7)	47.8 ± 1.8 (6)	<0.005
	23	30.2 ± 2.1 (6)	51.5 ± 2.0 (4)	<0.005	28.2 ± 3.2 (6)	44.3 ± 2.4 (7)	<0.005
	41–45	134.0 ± 10.4 (7)	193.0 ± 3.9 (9)	<0.005	113.0 ± 7.7 (6)	169.0 ± 5.2 (7)	<0.005
	70–71	313.0 ± 10.0 (10)	366.0 ± 8.7 (8)	<0.005	210.0 ± 10.0 (6)	233.0 ± 6.9 (7)	<0.05
Tail length (cm)	20–21	7.1 ± 0.2	8.1 ± 0.2	<0.005	7.3 ± 0.1	8.2 ± 0.2	<0.005
	23	6.9 ± 0.4	8.1 ± 0.2	<0.05	7.2 ± 0.3	7.4 ± 0.2	NS
	41–45	11.9 ± 0.5	14.2 ± 0.1	<0.005	11.6 ± 0.4	14.1 ± 0.2	<0.005
	70–71	18.4 ± 0.4	19.3 ± 0.4	NS	17.5 ± 0.3	17.9 ± 0.3	NS
Tibia length (cm)	20–21	2.07 ± 0.06	2.26 ± 0.03	<0.01	2.12 ± 0.03	2.21 ± 0.03	<0.025
	23	2.08 ± 0.04	2.30 ± 0.01	<0.005	2.08 ± 0.04	2.19 ± 0.03	<0.025
	41–45	3.08 ± 0.05	3.31 ± 0.02	<0.005	2.95 ± 0.05	3.22 ± 0.02	<0.005
	70–71	3.88 ± 0.04	3.96 ± 0.03	<0.05	3.61 ± 0.03	3.63 ± 0.06	NS

Note. Data are given as mean ± SEM. Numbers in parentheses are *N*. In each group the *N*'s for tail length and for tibia length are the same as the *N* for the corresponding body weight measurement.

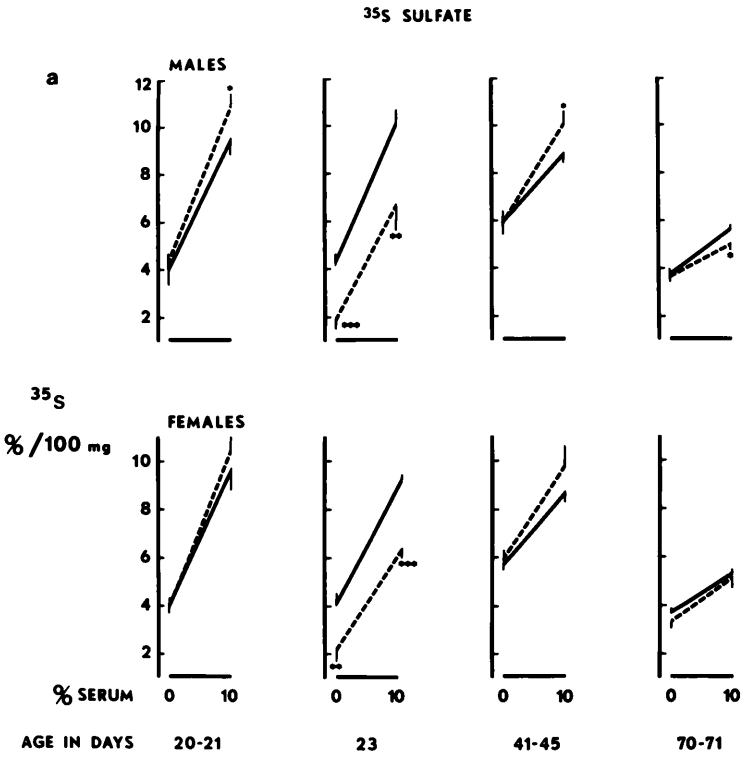

FIG. 1. *In vitro* incorporation of radioisotope by cartilage of head-irradiated rats (– – –) and of controls (——). The results of labeled substrates are shown in separate panels. (a) [^{35}S]sulfate; (b) [Me-^{3}H]thymidine; (c) L-[1-^{14}C]leucine; (d) L-[3,4-^{3}H]proline. The results of males and females are given separately. Vertical bars represent SEM. Significance according to one-tailed *t* test results are displayed as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Incubation with [Me-^{3}H]thymidine (Fig. 1b). At 20–21 days no significant difference in ^{3}H incorporation was found between irradiated and control cartilage in either medium. At 23 days significantly lower incorporation of ^{3}H was found in irradiated rats of both sexes in both media. At 41–45 days mean ^{3}H incorporation in irradiated males was significantly decreased in males in medium without NRS, but it was near the control level in medium with NRS. No difference existed between irradiated and control female rats at this age. At 70–71 days there was no effect of irradiation on ^{3}H uptake in either sex.

Incubation with L-[1-^{14}C]leucine (Fig. 1c). At 20–21 days, 41–45 days, and 70–71 days cartilage of irradiated rats of both sexes had higher mean incorporation of ^{14}C than did controls. These differences were significant at 20–21 days in males and in medium with NRS, at 41–45 days in both sexes and both media, and at 70–71 days in males in both media. At 23 days there was no significant difference between controls and irradiated rats.

Incubation with L-[3,4-^{3}H]proline (Fig. 1d). At 20–21 days cartilage of irradiated males and females had greater mean values for ^{3}H incorporation than did that of controls; these differences were significant for females in medium without NRS and for males in medium with NRS. There was no significant difference at 23 days. At 41–45 days significantly greater incorporation of ^{3}H occurred in irradiated rats of both sexes in both media. At 70–71 days mean values were consistently higher in irradiated rats than in controls, but the differences were not significant.

Discussion. The reduction in body weight and in tibial and tail length measurements of rats after neonatal head irradiation are consistent with the results of previous studies which showed almost complete absence of

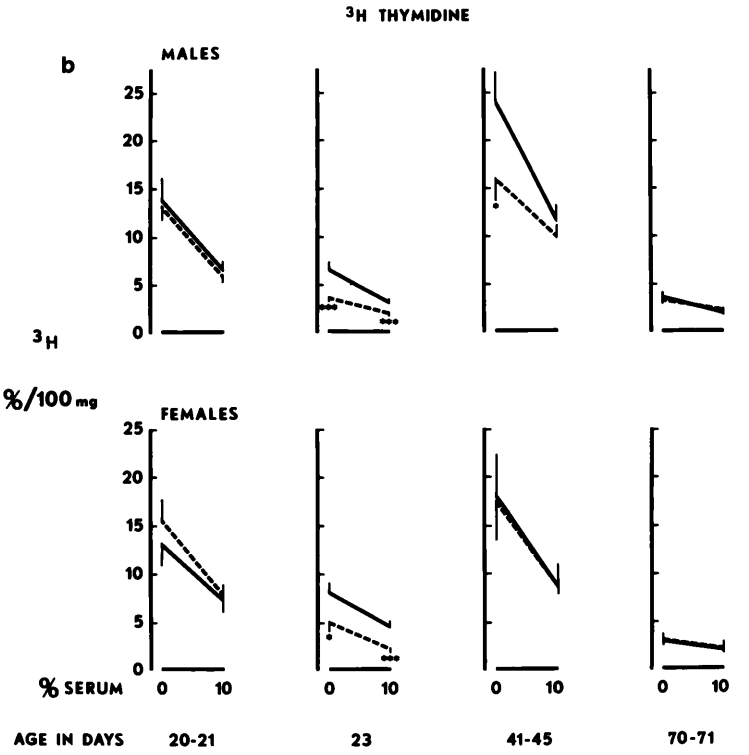

FIG. 1—*Continued.*

catch-up growth in irradiated male rats and only slow catch-up growth in irradiated female rats up to and including 121 days of age (3). In the present experiments only tibial and tail length in irradiated female rats, and tail length in irradiated male rats, underwent full catch-up growth. All other results excepting tail length in irradiated females at 23 days, showed significant growth impairment after neonatal head irradiation.

In vitro metabolism of cartilage in organ culture has been shown to correlate with the preexisting *in vivo* condition in hypopituitarism (12–14), primary hypothyroidism (15), glucocorticoid excess (16, 17), and starvation (18–21). Altered cartilage function *in vitro* has also been observed in rats during growth recovery after glucocorticoid excess (17) and after hypothyroidism (15). It is presumed that *in vitro* functions of freshly removed cartilage are influenced by residual growth factors derived from the circulation *in vivo* or produced within the cartilage itself. The utilization by cartilage of labeled sulfate, thymidine, leucine, and proline in the present experiments is interpreted to reflect synthesis of sulfated proteoglycans, DNA, chondroprotein, and collagen, respectively (11).

All four functions tested in the present experiments are known to be stimulated by somatomedin, a growth hormone-dependent peptide (11). Therefore, a lack of consistency in the observed response of these functions suggests action by other stimulators or inhibitors. The 41–45 days group showed an inconsistency in that cartilage of irradiated rats of both sexes had increased isotope incorporation after incubation with labeled sulfate, leucine, and proline, but not after incubation with labeled thymidine. The male rats showed, moreover, a significant decrease in thymidine incorporation in comparison to controls in buffer without added serum. This pattern can not be explained on the basis of increased somatomedin activity alone because of the lack of stimulation of thymidine incorporation. The observed pattern could result from presence in the cartilage of a hypothetical factor(s) stimulating cartilage functions with the exception of DNA synthesis or from concomi-

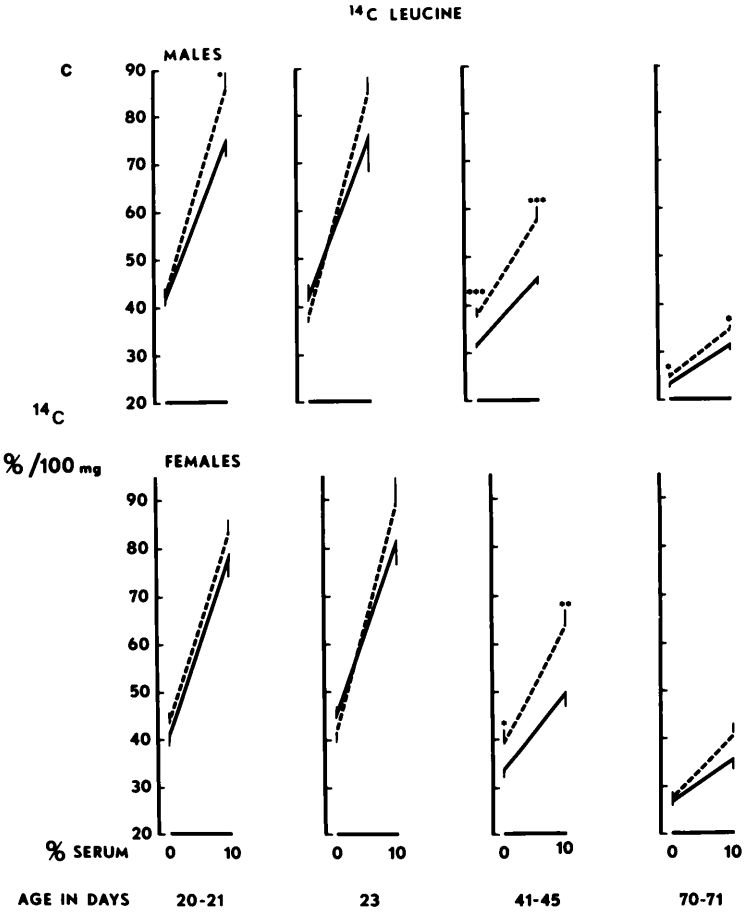

FIG. 1—*Continued.*

tantly increased activity of somatomedin and a selective thymidine inhibitor. The latter hypothesis is a more attractive possibility than the former because a selective thymidine inhibitor has been demonstrated in normal rat serum (23, 24). Indeed, the presence of this factor is shown again in the present experiments by the inhibition of thymidine incorporation in all the cartilage groups incubated in medium with NRS.

The decreased incorporation of labeled sulfate and thymidine in irradiated rats at 23 days of age probably resulted from transient undernutrition (20, 21) at weaning caused by radiation-induced neurological injury resulting in awkwardness and uncoordination. It is unlikely, however, that the enhanced sulfation in males at 41–45 days of age is related to previous undernutrition, because earlier studies have shown that *in vitro* sulfation in rat costal cartilage does not rise above normal values during catch-up growth following periods of undernutrition (21). At other time periods, 20–21 days and 70–71 days, the metabolic functions of cartilage differed only slightly and, for the most part, insignificantly between irradiated and control rats, thus excluding a state of undernutrition at those times.

The observed metabolic pattern in the irradiated rat cartilage at 41–45 days has prompted us to begin ultrastructural studies of costal and tibial cartilage in similarly treated animals. Preliminary results (unpublished) indicate increased aggregation of collagen fibrils, enhancement of cell degeneration, increased numbers of matrix vesicles, and premature cartilage calcification. The findings are

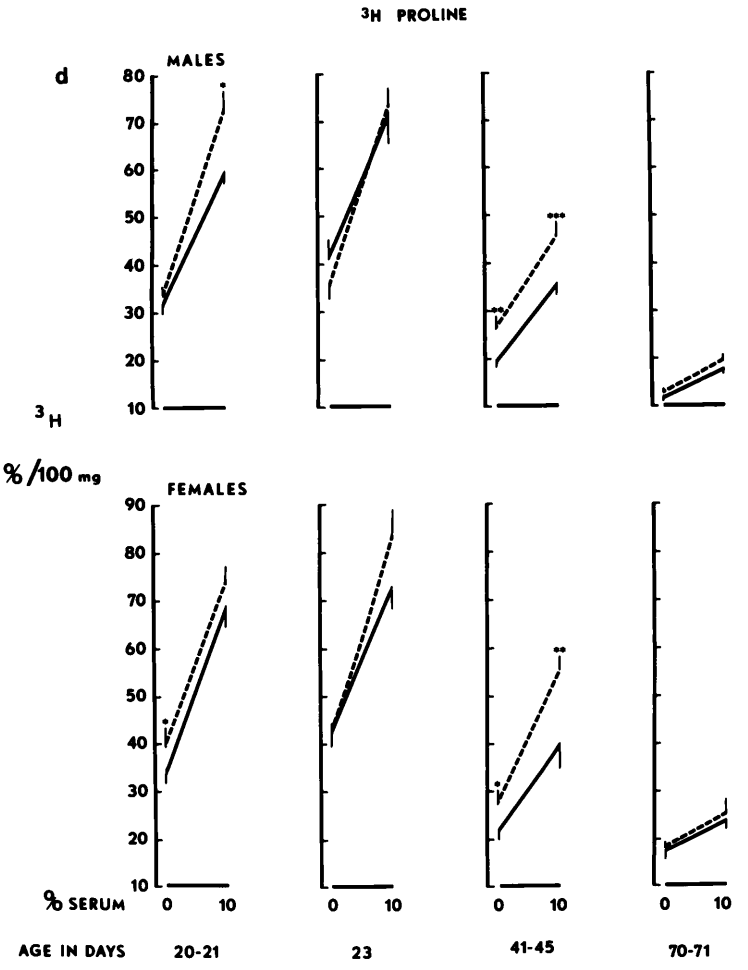

FIG. 1—*Continued.*

consistent with ultrastructural changes of cartilage during the recovery period after fasting (25, 26) hypothyroidism (26), and glucocorticoid treatment (27–30) as well as those associated with aging in rats and in humans (31–33).

We conclude that head irradiation of the neonatal rat produced a delayed abscopal effect on the metabolism of developing cartilage. The pattern of the metabolic effect suggests that it is in part produced by a factor(s) other than somatomedin.

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