Effect of 2000 Roentgens Local X-Irradiation on Metabolism and Alkaline Phosphatase Activity of Rat Bone

S. H. COHN AND J. K. GONG

From the Clinical Radiobiology Branch, United States Naval Radiological Defense Laboratory, San Francisco, California

It is the object of the present investigation to study some of the quantitative effects of radiation on bone growth and calcium metabolism, and to investigate the mechanism by which radiation produces these effects on bone. In view of the fact that ionizing irradiation of living animals results in disturbances in normal bone growth (1–5) and further, that there is a close relationship between alkaline phosphatase and calcification (6–9), an investigation of this enzyme in the bones and serum was undertaken. Particular attention was placed on the relationship between alkaline phosphatase activity and bone matrix formation in normal and irradiated animals.

It has been found in previous experiments that whole body x-irradiation of young rats with 500 roentgens produced only temporary bone damage (10), which was reflected in a decrease in the rate of growth of tibia weight in the irradiated animals, a diminution in alkaline phosphatase activity of the tibia, and a marked decrease in the serum alkaline phosphatase.

The marked changes in bone resulting from x-ray treatment, which are described in the literature, occur after much larger doses than the relatively small dose of 500 roentgens total body irradiation affords locally. In order to increase the applied dose to the bone without producing generalized harmful effects involving the remainder of the animal, it was decided to administer a dose of 2000 roentgens x-irradiation to one hindleg.

EXPERIMENTAL

These experiments were designed so that one tibia of each of a large group of young rats of uniform age was treated with 2000 roentgens x-irradiation. These animals were then followed up to adult life and the growth and calcification of both tibiae and the tibiae of a similar group of control animals were then studied, measurements being made at regular intervals under standardized conditions.

In *experiment 1*, 120 animals were used, 60 of which received the x-irradiation and 60 of which were kept as nonirradiated controls. This experiment was repeated at a later date with 54 animals, all of which were irradiated (*experiment 2*). (Because it was noted that there were large variations in the body weights of the animals of *experiment 1*, the animals of *experiment 2* were especially selected so that the weights fell reasonably close to the average value for the age.) The animals used were male Sprague-Dawley rats. The animals in the 1st experiment were approximately 41 days of age at the time of irradiation while the animals of the 2nd experiment were approximately 39 days of age.

Six animals were placed in each experimental group, and 6 animals in each control group, for each of the ten times of killing in *experiment 1*. Six animals were placed in each group for each of the nine times of killing in *experiment 2*.

The animals were anesthetized and placed in a lead cylinder with the left hindleg protruding. Each of the experimental animals was exposed to 2000 roentgens over the left hindleg. The field of irradiation covered the distal end of the femur, all of the tibia-fibula, and the entire foot. The remainder of the animal was protected with ¼ in. of lead shielding.

Dosage factors used were: 250 kvp, 15 ma, 35 cm target-to-skin distance, 9.3 min exposure, 0.5 mm Cu/mm Al.

The animals were placed 2 in a cage, and fed ad libitum throughout the experiment. Food and water were withheld for the 20 hr preceding killing.

Serum Alkaline Phosphatase. Blood samples were obtained under Nembutal anesthesia by the open chest method of cardiac puncture. The serum was analyzed for phosphatase activity using a modification of the method of King and Armstrong (11). The values for phosphatase activity represent mg phenol liberated in 30 min from a substrate of disodium phenyl phosphate at 37°C. The buffer used was diethylbarbiturate, which enabled reactions to be carried out at pH 9.3. A Beckman Spectrophotometer (Model DU) was used to read the optical densities at 600 mμ.

Weight of Matrix of Tibia. Both right and left tibiae were removed immediately after killing. They were dissected clean, and weighed. The bones were homogenized (as described below), made up to 25 ml and allowed to stand at room temperature for 24 hr. An aliquot of the solution was removed for the analysis of the alkaline phosphatase. The remainder of the solution was put in a drying oven at 100°C and left

Received for publication November 26, 1952.

Downloaded from http://ajplegacy.physiology.org/ by 10.220.33.2 on January 24, 2017

there overnight. The dry residue was weighed (dry weight) and after being placed in a muffle oven set at 500°C overnight, again weighed (ash weight). The difference between the dry weight and the ash weight is taken as the weight of the matrix, representing the dehydrated organic portion of the bone.

Tibia Alkaline Phosphatase. Following extraction of the enzyme from the tibia, the method of estimation of the concentration of the alkaline phosphatase in bone was essentially the same as in estimating serum alkaline phosphatase.

To prepare homogenates of the tibiae for the enzyme determination, the bones were first frozen with liquid nitrogen, and then placed in a specially constructed steel mortar which was precooled. This apparatus was designed for a precision fit between the pestle and mortar. The bones were struck with a steel pestle until the bone fragmented into small pieces. The fragments were transferred to a 25 ml volumetric flask and diluted to mark with water. Several drops of chloroform were added as a preservative, and the suspension was allowed to autolyze at room temperature for 24 hr, with occasional shaking. At the end of the extraction period, the suspension was filtered, and aliquots taken. Sodium carbonate-bicarbonate buffer was used, which allowed the reaction to proceed at pH 10.1. The measure of the reaction velocity thus attained was divided by the ash weight of the tibiae, to obtain the measure of reaction velocity/100 mg ash weight of tibia.

Uptake of Intraperitoneally Injected Calcium^{45} Chloride (Used as a Measure of Rate of Calcification). Radiocalcium, in the form of calcium chloride ($Ca^{45}Cl_2$) was used in this study of the formation of growing bone to determine the rate of uptake of calcium by the bones from the blood.

The rats were injected intraperitoneally with a tracer dose of carrier-free radiocalcium [0.25 ml (7.5 μc) of $Ca^{45}Cl_2$] at 20 hr preceding killing. The amount of radioactivity present in this dose of Ca^{45} is small enough to have no damaging effects due to irradiation. Further, the calcium content of this dose is sufficiently small to behave like the amounts normally entering the circulation.

At each killing interval, aliquots of the homogenates made of the tibia, as described above, were dried in an oven at 100°C and ashed at 500°C in an electric furnace. The ash was dissolved in 2 N HCl and an aliquot was then evaporated onto a copper disc. The activity was counted under a thin end window Geiger Muller counter connected to a standard scaling circuit. An injection solution standard was prepared in the same manner after suitable dilution. The activity readings were corrected for any possible self-absorption effects by the use of suitable control samples, and the percentage of the injected dose present in the tibia was calculated.

TABLE 1. SUMMARY OF DATA OF EXPERIMENT 1

Day	Tibia Matrix, mg			% Uptake Ca^{45} /gm Tibia Ash			Units Tibia P'tase /gm Ash			Units Serum P'tase /100 ml Serum	
	C	XR	XL	C	XR	XL	C	XR	XL	C	X
1	86	74	81	30.8	33.6	33.8	13.2	12.2	9.5	48.5	42.4
3	86	101	86	29.2	30.5	31.2				50.2	39.8*
7	100	102	100	26.4	22.3	20.9	11.6	10.2	7.0*	48.2	31.8*
11	116	124	113*	19.2	18.0	14.9*	8.3	7.3	4.7*	42.4	36.1*
15	108	107	97*	17.7	18.0	12.3*	10.0	8.8	4.5*	47.6	33.1*
22	124	131	109*	16.4	15.6	13.4*	9.1	9.2	7.2*	51.5	30.1*
29	137	158	115*	15.6	15.5	12.2*	7.7	5.8	5.1	41.1	30.2*
60	143	155	105*	8.9	9.6	7.1*	5.6	4.8	2.3*	23.9	18.5*
90	192	189	136*				4.5	3.9	2.2*	34.3	18.9*
120	202	184	131*	6.0	6.5	4.6*	2.4	2.5	1.4	34.6	21.5*

C, Control animal, left tibia. *XR*, Experimental animal, right tibia, nonirradiated. *XL*, Experimental animal, left tibia, irradiated with 2000 r. * The 'T' value shows a statistically significant change at the 95% level.

Each value represents the mean for 6 rats.

RESULTS

The local effects of irradiation were observed by comparing the matrix weight, calcium^{45} uptake and tibia alkaline phosphatase levels of the irradiated tibiae with the nonirradiated homologous tibiae. Systemic effects to the nonirradiated tibia were observed by comparing the above measurements in the nonirradiated homologous tibia of the irradiated animals (homologous control tibia), with the tibia of the normal nonirradiated animals (control tibia). These values are summarized in tables 1 and 2. The control tibiae and the homologous control tibiae exhibit no significant difference in the matrix weight, Ca^{45} uptake or tibia alkaline phosphatase activity throughout the period of the experiment. One nonlocal effect was observed, namely, the serum alkaline phosphatase of the irradiated group fell below the phosphatase values for the controls on the 1st day postirradiation, and remained lower than the controls to the conclusion of the experiment. The amount of depression remained relatively constant during this period.

Downloaded from http://ajplegacy.physiology.org/ by 10.220.33.2 on January 24, 2017

It is to be noted that the serum phosphatase of the control animals fell off gradually from 40–160 days of age.

The weight of the organic portion of the bone, or matrix, in the irradiated tibia does not differ significantly from the weight of normal matrix for the first 10 days. After this time the rate of growth of the irradiated matrix decreases until the end of the period of observation.

Metabolic Effects. The values for Ca^{45} uptake at various intervals of time after irradiation are shown in the tables. In the control tibiae the values for the uptake of Ca^{45}/gm tibia ash fall off gradually as the animal grows to maturity. No difference in the radiocalcium uptake was detected in the homologous controls as compared to the control tibiae.

In the irradiated tibiae, the Ca^{45} uptake/gm ash follows the curve of the control tibiae for the first 3 days, and then drops, reaching a minimum at 15 days. There is a partial recovery in the period 15–30 days, and following this, the Ca^{45} uptake remains at a relatively constant depressed level for the remainder of the experiment.

Tibia Alkaline Phosphatase Activity. It is to be noted that, as the animals gain maturity, the tibia phosphatase activity decreases, with the adult animals showing a value of phosphatase activity in their bones one-fourth of that in the bones of young growing animals. Again, no significant difference was found to exist between the controls and the homologous controls.

The phosphatase activity of the irradiated tibiae is sharply lowered in the period 1–15 days, and exhibits a partial recovery in the period 15–22 days. By 90 days, the phosphatase activity of both the control tibiae and the irradiated tibiae fell to values less than 3 U/100 mg ash.

In the nonirradiated legs the tibia phosphatase concentration is related to tibia matrix weight, as is shown in figure 1.[1] Here the log of matrix weight is plotted against tibia phosphatase/100 mg ash and the following linear function is obtained: $\log M = \log 266.2 + P \log 0.91$. P is the tibia alkaline phosphatase activity expressed in King-Armstrong U/100 mg ash and M is the weight of the matrix in mg. No significant difference is observed between the values for the controls and those for the homologous controls. The slopes and intercepts for the lines of regression in the above equation, relating the matrix weight and tibia alkaline phosphatase concentration, were determined on the basis of all the control values. The coefficient of correlation (Brevais-Pearson) relating the 2 variables was found to be 0.95. The values for the irradiated tibiae do not appear to follow a single exponential function as do the controls. In addition, the values for the irradiated tibiae have a considerably greater spread than do those of the controls. This is probably due to the variation in radiosensitivity of the tissues of different animals.

TABLE 2. SUMMARY OF DATA OF EXPERIMENT 2

	Tibia Matrix, mg		% Uptake Ca^{45}/gm Tibia Ash		Units Tibia P'tase/100 mg Ash	
Day	XR	XL	XR	XL	XR	XL
1	69.1	69.3	40.8	42.0	13.3	11.4
3	75.9	77.6	37.5	37.8	16.4	9.3*
7	87.6	86.8	27.9	20.5*	13.0	7.4*
15	86.8	71.1*	20.7	12.3*	9.6	4.3*
22	118.0	92.0*	14.9	10.9*	8.5	5.8*
29	140.0	114.0*	13.8	9.7*	—	4.8
60	161.0	128.0*	7.9	7.3	5.1	2.7*
90	196.0	151.0*	8.4	5.8*	3.5	3.4
120	183.0	129.0*	6.9	5.8*	2.7	2.9

XR, Experimental animal, right tibia, nonirradiated. *XL*, Experimental animal, left tibia, irradiated with 2000 r. *The T value shows a statistically significant change at the 95% level.

Each value represents the mean for 6 rats.

DISCUSSION

A nonlocal effect observed was the lowering of the serum alkaline phosphatase level. It may be presumed from this that bone is one of the contributors to the serum alkaline phosphatase.

Since no significant difference appeared between the Ca uptake and the alkaline phosphatase activity of the homologous control tibiae and those of the tibiae of the nonirradiated control animals, it must be assumed that there is no systemic effect upon these

[1] In this figure, only the mean values for each day of the experiment (average of 6 values) are shown for clarity.

Downloaded from http://ajplegacy.physiology.org/ by 10.220.33.2 on January 24, 2017

functions of the nonirradiated tibiae of the animal following irradiation of the other tibia.

As the skeletal tissue is capable of withdrawing Ca^{45} from the circulating blood and of fixing small numbers of the labeled atoms, the measure of Ca^{45} uptake reflects the rate of normal bone formation and resorption.

As would be expected, the young rapidly growing animals have a more rapid calcium^{45} bone uptake and reach a higher specific activity than do the mature animals. The gradual decrease in the avidity of the tibia for Ca^{45} as the animal grows to maturity is correlated with the decreased rate of bone

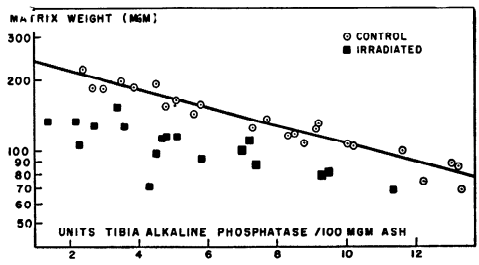

FIG. 1. Effect of 2000 roentgens local x-irradiation on the tibia alkaline phosphatase concentration per matrix weight.

growth and with decreased osteoblastic activity. These data on Ca^{45} uptake are similar to that of Newman (12) on the rate of recrystallization of bones. He found that the slow incorporation of the $P^{32}O_4$ ion by the mineral crystals declines with the increasing age of the crystal. The age of the crystal, in turn, is shown to be a function of the age of the animal (12). Here the similarity between the effect of irradiation and that of aging on bone metabolism may be seen. The inability of the irradiated bone tissue to fix radiocalcium as well as does normal bone indicates a profound difference between the metabolism of the irradiated and normal tibiae.

It was observed in the growing male rats that the level of tibia alkaline phosphatase per unit weight of tibia falls off gradually as the animal reaches maturity, thus correlating with the decreased rate of growth. The tibia phosphatase concentration is linearly related to the log of the matrix. Tibia alkaline phosphatase appears, therefore, to be a good index of bone growth.

Following a massive dose of x-irradiation, the tibia alkaline phosphatase level is sharply depressed and reaches a minimum value at a time which coincides with the period of the greatest retardation in bone growth. The depression of alkaline phosphatase in particular, appeared to be correlated with the decrease in the tibia matrix weight resulting from x-irradiation. Histological observations of Siffert (9) have indicated that the elaboration of bone matrix is a function of alkaline phosphatase. Bourne (13) has also pointed out that the reduction of phosphatase activity observed in cases of scurvy appear to be correlated with the reduction in bone matrix. Interestingly, the ratio of bone phosphatase concentration to matrix in both scorbutic bone and in irradiated bone does not differ from that found in normal bone.

As the depression of growth continues long after the short period of irradiation, the effect of irradiation on growth can be analyzed in terms of destruction of growth capacity. Since the results indicate that the growth capacity for the tibiae is, at least in part, within the bone itself, it must be determined by one or more of the cellular components of bone. The diminution in growth capacity thus would result indirectly from the destruction of these cells by irradiation.

According to Bloom (14), a significant histological indication of bone damage following exposure to large doses of x-radiation is the disappearance of osteoblasts. Osteoblasts have long been known to be associated with the formation of osseous tissue, and appear on the surface of bone as long as active growth occurs (15). The osteoblasts have also been shown to be associated with the production of alkaline phosphatase. For example, Gould and Schwachman (16) concluded from histological evidence that osteoblasts are the source of phosphatase, and further, that the phosphatase activity of bone appeared to reflect the osteoblastic activity in scurvy. In addition, Franseen and McLean (17) also provide evidence for the theory that phosphatase is synthesized by the osteoblasts, in that there is an increased production of phosphatase in osteoblastic types of osteogenic sarcoma. These sarcomata showed a fall in plasma phosphatase after x-irradiation.

Downloaded from http://ajplegacy.physiology.org/ by 10.220.33.2 on January 24, 2017

It was found that injected radium (18), a bone seeker, which delivers a high specific ionization to bone results in a destruction of osteoblasts and a depression of bone phosphatase activity. However, when solutions containing bone phosphatase were irradiated with 10,000 r no effect on the enzyme activity was obtained (18). This is evidence that the reduction in the alkaline phosphatase activity following x-irradiation of bone is due to damage to the source of the phosphatase in the skeletal tissue, and not to direct inactivation of the enzyme itself by the irradiation.

From these relationships, an explanation for the depressing action of radiation on the growth and metabolism of bone may be proposed. Local x-irradiation of bone, with a sufficiently high dose, causes a destruction of various cellular components, especially of the osteoblasts. Since the osteoblasts are most probably the precursors of tibia alkaline phosphatase, the level of the enzyme in bone falls in proportion to the number of cells destroyed. The depression of alkaline phosphatase level and thus activity results in the loss of its vital functions in bone growth. These functions most likely include a role in the formation of bone matrix as well as its previously established role in calcification.

SUMMARY

It was found that a dose of 2000 roentgens administered to one leg produced chiefly local effects in the skeletal tissue of the young growing rats. A nonlocal effect observed was a depression of the serum alkaline phosphatase. This would indicate that the bone is probably one of the sources of serum alkaline phosphatase. The matrix weight, calcium^{45} uptake and alkaline phosphatase activity of the irradiated tibia were compared with the corresponding measurements in the homologous nonirradiated tibia in the same animals. No significant differences in any of the above measurements were found between the homologous control tibiae and the tibiae of nonirradiated animals. The rate of calcium^{45} uptake, measuring the rate of calcification was found to be depressed following 2000 r x-irradiation directly to one leg. The depression of calcium metabolism following irradiation is similar to that found in the normal aging bone. It was observed that tibia alkaline phosphatase activity concentration decreases as the animals grow to maturity. Further, following 2000 r x-irradiation, the phosphatase enzyme level was sharply depressed, correlating quite closely with the depression of bone growth. In normal animals, tibia alkaline phosphatase concentration was found to be linearly related to the log of the matrix weight. The depressing action of irradiation on the growth and metabolism of bone may be due primarily to the depression of alkaline phosphatase activity resulting from the destruction of the osteoblasts which are presumably the precursors of the enzyme in bone.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

REFERENCES

1. Bisgard, J. D. and H. B. Hunt. *Radiology* 26: 56, 1936.
2. Regen, E. M. and W. E. Wilkins. *J. Bone & Joint Surg.* 18: 61, 1936.
3. Brooks, B. and H. T. Hillstrom. *Am. J. Surg.* 20: 599, 1933.
4. Hinkel, C. L. *Am. J. Roentgenol.* 47: 439, 1942.
5. Cohn, S. H. and J. K. Gong. *Growth* 17: 7, 1953.
6. Robison, R. *The Significance of Phosphoric Esters in Metabolism.* New York: New York Univ. Press, 1932, pp. 18.
7. Gomori, G. *Am. J. Path.* 19: 197, 1943.
8. Lorch, I. J. *J. Bone & Joint Surg.* 31: 94, 1949.
9. Siffert, R. S. *J. Exper. Med.* 93: 415, 1951.
10. Cohn, S. H. and J. K. Gong. Effect of 500 roentgen whole body x-irradiation on the growth and metabolism of rat bone. San Francisco, Calif.: U. S. Nav. Def. Lab., 321, pp. 21.
11. Binkley, F., R. H. Shank and C. L. Hoagland. *J. Biol. Chem.* 156: 253, 1944.
12. Newman, W. F. and D. J. Mulryan. *The Surface Chemistry of Bone. vi. Recrystallization in vivo.* Rochester, N. Y.: Univ. of Rochester, 184, pp. 15.
13. Bourne, G. H. *J. Physiol.* 102: 319, 1943.
14. Bloom, W. *Histopathology of Irradiation From External and Internal Sources.* New York: McGraw-Hill, pp. 70, 1948.
15. Maximow, A. A. and W. Bloom. *A Textbook of Histology* (4th ed.). Philadelphia: Saunders, pp. 695, 1942.
16. Gould, B. S. and H. Schwachman. *Am. J. Physiol.* 135: 485, 1942.
17. Franseen, C. C. and R. McLean, *Am. J. Cancer* 24: 299, 1935.
18. Norris, W. P. and S. H. Cohn, *J. Biol. Chem.* 196: 255, 1952.

Downloaded from http://ajplegacy.physiology.org/ by 10.220.33.2 on January 24, 2017