THE DISTRIBUTION OF PHOSPHORUS IN SOME BONES OF THE WHITE RAT (RATTUS NORVEGICUS ALEINUS) WHOSE GROWTH HAS BEEN ACCELERATED BY GROWTH HORMONE.

I. NINETEEN HOURS AFTER A SINGLE INJECTION OF RADIOACTIVE PHOSPHORUS

by

ROBERT HENRY BUCIIOLZ

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INTRODUCTION AND REVIEW OF LITERATURE

The bones serve not only as structural elements but also as sites of calcium and phosphorus reserves which may be drawn upon at times when the assimilation of these minerals is inadequate to meet body needs. Thus the mineral metabolism of bone involves not only the deposition of calcium and phosphorus during growth but also processes of storage and mobilization which occur throughout life.

In typical long bones such as the femur and humerus the diaphysis or shaft consists of a somewhat cylindrical mass of compact bone surrounding a central cavity filled with fatty bone marrow. The epiphysis, at each end of the shaft, consists of spongy bone with a thin peripheral cortex of compact bone. In the growing animal the epiphysis and the diaphysis are separated by the epiphyseal cartilage plate, which is united with the relatively compact bone of the diaphysis by trabeculae of spongy bone, often called the metaphysis. The epiphyseal cartilage, together with the spongy bone of the metaphysis, forms a growth apparatus responsible for the growth in length of the long bones. When the cells of the epiphyseal cartilage plate cease to multiply, the cartilage becomes entirely replaced by bone, the epiphysis unites with the diaphysis, and growth ceases. This is referred to as the closing of the epiphyses. Bones are covered with a modified connective tissue, the peristeam; a somewhat similar tissue, the endosteam, lines the marrow spaces,
including those in spongy bone.

There are two types of bones based upon the mode of formation. One type of bone develops through the transformation of connective tissue (intramembranous ossification). The second type develops by a replacement of cartilage (intracartilaginous or endochondral ossification). Both cartilage and membrane bones grow in thickness through the progressive deposition of periosteally-formed bone immediately under the periosteum and thus upon the outer surfaces of the bone proper.

As distinguished from the compact bone of the diaphysis, the meshwork of trabeculae of the spongy bone constitute a reserve of calcium and phosphorus which may be mobilized to meet needs not currently supplied by the diet. This function of the trabeculae has been clearly portrayed by the detailed studies of Bauer, Aub, and Albright (1929). It is of interest to note the trabeculae are most numerous at the epiphyseal ends of the bones where the blood supply is greatest. The depletion of the trabeculae of mineral salts during periods of rapid growth for the building of the structural portion of the bone involves no physiological harm, and their mineral content is readily restored with an adequate diet during periods when the body needs for these elements are less.

Approximately 30 per cent of the total phosphorus of the body is present in the bones and teeth. Though somewhat variable according to age, state of nutrition, and species, normal adult bone may be considered to have the following approximate
composition: water, 45 per cent; ash, 25 per cent; protein, 20 per cent; and fat, 10 per cent. In mammals the ash consists approximately of the following: calcium, 36 per cent; phosphorus as phosphate, 17 per cent; carbon dioxide as carbonate, 5.5 per cent; and magnesium, 0.8 per cent. The rest is made up of small amounts of other minerals.

The mechanism of the deposition of phosphorus in bone is still incompletely understood. At least two factors, local and humeral, are involved. The humeral factor is related to the supply of minerals in the fluids of the body, and to the solubility of the calcium salts; it may be defined in terms of the concentrations of these substances in the blood. The local factor is not well understood. It determines the occurrence and specific localization of the deposition of bone salts, when adequate concentrations of mineral salts are present in the blood.

The interstitial substance of bone acts as a calcium depot. A constant interchange of this substance takes place between the blood and the bones, with the result that the calcium ion concentration in the plasma remains fairly constant. Calcium, once deposited in the form of bone salts, may be made available to the blood only by destruction of osseous tissue, including its organic matrix, chiefly from the spongy bone near the epiphyses of the long bones. The rate of the resorptive process is regulated by the parathyroid hormone, which however, has no effect upon the deposition or calcification of bone.
The growth of bone is markedly influenced by the growth hormone of the anterior pituitary. Silberberg and Silberberg (1941) state that during the processes of skeletal growth and ageing, three main phases may be distinguished: (a) During the first period proliferation of the epiphyseal cartilage predominates, producing lengthwise growth of the long bones. Towards the end of this period proliferative processes decline and the epiphyseal discs narrow gradually. Simultaneously periosteal growth processes cause an increase in the thickness of the bone. (b) During the second period regressive changes, such as atrophy, disintegration, and calcification of the epiphyseal cartilage predominate. These changes, associated with, or followed by, ossification, coincide with the cessation of elongation of the bone and with a further increase in the density and thickness of the diaphysis. (c) During the third period, ossification of the epiphyseal cartilage progresses, but at the same time processes of resorption of the bone by elements of the bone marrow and of the periosteal tissue begin to predominate. This leads to a thinning of and to more or less extensive perforations of the epiphyseal discs and to a thinning of the shaft of the long bones. The authors also found that when growth hormone was injected subcutaneously, proliferation of cartilage was temporarily increased, upon which it ceased prematurely. The processes of degeneration of cartilage and deposition of bone likewise set in earlier and proceeded more rapidly and more intensely than usual, as did the processes of resorption. According to
Ross and McLean (1940), in rats six months of age, injections of anterior pituitary growth hormone were ineffective when given intraperitoneally, but stimulation of growth of cartilage was observed if the injections were given subcutaneously.

Overactivity of the anterior lobe of the pituitary gland (hyperpituitarism) may occur either during childhood, before the bones have attained their full length, or any time during adult life after union of the epiphysis and diaphysis has taken place and bone length has been fixed. If overactivity of the anterior lobe of the pituitary occurs during adult life, the bones cannot further increase in length but sub-periosteal ossification may take place resulting in a thickening of the bones which is especially noticeable in the hands, feet, and face. This enlargement of the extremities is known as acromegaly. When hyper-pituitarism takes place in childhood or early adolescence the height of the body is increased before the closure of the epiphyses and gigantism results.

Gigantism was first induced experimentally in the rat by injection of extracts from the anterior pituitary gland by Evans and Long (1921). A growth promoting hormone in the extract was postulated as the cause of the excessive growth. During the years in which this laboratory did work, the growth hormone was gradually freed of other contaminating principles found in the anterior lobe and the phenomenon of an overgrowth of the normal body was steadily confirmed (Evans, Simpson, and Li, 1943). However, doubt arose as to the existence of a separate pituitary
principle responsible for growth, partly due to the fact that in some animals other pituitary principles appeared to affect growth. Furthermore, it was found that with daily administration of pituitary extracts growth was sometimes not continuous, and the possibility of the development of a specific anti-growth factor was discussed. With the isolation of the pure growth hormone (Li, Evans, and Simpson, 1945) further repetition of the continuous administration of the principle became necessary, in order to establish the biological properties of this hormone when freed from other anterior hypophyseal substances. Evans, Simpson, and Li (1948) found that continued daily injection of growth hormone into normal "plateaued" female rats resulted in continuous growth for a 437 day period, or until the animals were 647 days of age. The rate of growth was not, however, as rapid at the end of the period as at the beginning. The weights of viscera increased markedly and maintained the same proportion to the body weight as in the controls. The reproductive organs decreased in weight and thus constituted a much smaller percentage of the body weight than in controls. The endocrine organs all weighed less in proportion to body weight than in the normal controls. The adrenals and pituitaries were slightly increased in absolute weight, the thyroids slightly decreased. Histologically the thyroids, adrenals and ovaries showed no evidence of specific hormonal stimulation. The ovaries appeared to be ad-

1Plateaued refers to rats whose growth curve has leveled off.
versely affected in the injected rats.

Evans et al. (1948) illustrated that rats injected with growth hormone were significantly longer than controls; both trunk and tail contributed to this greater length, the proportion of tail length to body length not being changed by injection. The injected animals had larger skulls and greater size of the thoracic cage, pelvis, vertebral column, tail, and long bones. Skeletal growth was still continuing, though slowly, at the end of the 437 day period of daily injection of growth hormone. This was shown histologically in the proximal epiphysis of the tibia by the persistence of a zonal arrangement within the cartilage plate, the presence of some capillary erosion, and the formation of delicate trabecular bone. The corresponding region in the controls showed marked senescent atrophy.

The earlier work of Teel and Watkins (1929), indicated that anterior pituitary extract produced an increase of inorganic phosphorus in the blood of dogs. Faehl and Price (1937), working with dogs, observed that the inorganic phosphorus of the urine fell markedly following a single injection of a pituitary growth preparation. In rats, the removal of the pituitary reduces appreciably the inorganic phosphorus of the blood serum (Anderson and Oastler, 1933; Jones and Shinowara, 1942). The clinical data of Reifenstein, Kinsell, and Albright (1946), showed that the serum inorganic phosphorus level was elevated in both acromegalic patients and growing children. Li, Geschwind and Evans (1949), found that the inorganic phosphorus level in
the rat plasma decreased with age. Hypophysectomy caused a decrease of the plasma inorganic phosphorus content, while injections with growth hormone prevented this fall and elevated the phosphorus level above that of the control.

Manly and Bale (1939) state that quantitative data on radioactive phosphate deposition and turnover in normal animals are valuable because of the following applications: (1) as evidence for the mechanism and rates of normal calcification, (2) as a standard for comparison with variations produced by pregnancy, rickets, or diet, and (3) as an indication of the therapeutic possibilities of the B-ray emission of the isotope, based on information concerning its distribution in the animal. These authors found that a rapid deposition of blood phosphorus takes place in the bones. The epiphyses acquired about twice as much of the marked phosphorus per gram of inorganic tissue as the diaphyses in the first day following administration. This extends and confirms the work of Hahn, Nevesy, and Lundsgaard (1937). Manly and Bale (1939) also showed that the diaphyses had greater retention of acquired phosphorus after the labeled phosphorus in the blood had fallen to negligible amounts.

Pecher (1941) in experiments on the deposition of radioactive isotopes of calcium and strontium, demonstrated that the distribution of these two elements in body tissues was similar. Marx and Reinhardt (1942) found that hypophysectomized rats treated with growth hormone and consequently in a state of rapid growth, deposited in the femur and mandible, when injected with
radioactive strontium, essentially the same amounts of strontium as did untreated hypophysectomized control rats injected simultaneously with the same amount of radioactive strontium. Comparison of the injected and controls animals was made by determining the total radioactivity in two separate piles of ground-up bone.

The object of this investigation was to ascertain if growth hormone injected into normal rats would affect the deposition of radioactive phosphorus which was also injected. The problem, carried on jointly with the Department of Physics, is in the nature of a preliminary investigation of the role of growth hormone in accelerating growth of bone.

MATERIALS AND METHODS

White Wistar rats were used in this experiment. These rats had been interbred for several generations.

Purina Dog-Chow in the checkered form constituted the bulk of the diet. This food contained all of the necessary nutrients for normal growth. Small quantities of lettuce added to the diet of pregnant animals aided materially in the health of the litter.

Ten rats were divided into comparable groups and subdivided for the purpose of injection with the substances used in this experiment. Group A, containing three rats (No. 1, No. 2, and No. 3), was injected with both growth hormone and the radio-
active phosphorus. Group B, containing three rats (No. 4, No. 5, and No. 6), was injected with growth hormone. Group C, containing two rats (No. 7 and No. 8), was injected with radioactive phosphorus. Group D, containing two rats (No. 9 and No. 10), was not injected, and the animals were used as controls.

The animals were colored with combinations of alcoholic solutions of picric acid, mercurochrome, methyl green, and gentian violet, one of the colors being placed on the lumbar region and another color on the head. The males and females were separated in these groups so comparison could be made among individuals of the same sex.

The rats of Groups A and B were injected with growth hormone daily, except Sundays, from March 2, 1950, until they were sacrificed.

At first, one rat unit of the growth hormone per day was injected into each rat. When no striking differences in weight were apparent, the dosage was increased to two rat units on March 15. Three days later the dosage rate was still further increased to four rat units per rat per day. Fifty-two days later the dosage rate was 20 rat units of growth hormone per rat per day, a dosage which was maintained until the animals were sacrificed.

Weights and tail lengths (anus to tip of tail) were taken twice a week of all the animals of these groups. The record of the average weights of the rats is shown in Table 1. Fig. 1 shows the growth curves as averages of the males receiving the
Table 1. Record of the average weights of the rats.

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<th>Injected males</th>
<th>Normal females</th>
<th>Injected females</th>
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<th>Av. of all injected rats</th>
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* Injected male rats.
× Normal male rats.
* Injected female rats.
+ Normal female rats.

Fig. 1. Growth curves.
Fig. 2. Average growth curves.

* Injected rats.

x Normal rats.
hormone (Group A), the males not receiving the hormone, the females receiving the hormone, and the females not receiving the hormone. A comparison of the average weights of all rats injected with growth hormone and the average weights of all rats not injected with growth hormone is shown by Fig. 2. These results are discussed later.

Four grams of powdered growth hormone containing two thousand rat units were generously supplied by the Parke-Davis Company, Inc. The growth hormone was prepared for injection by dissolving a quantity of the powdered hormone in a sodium hydroxide solution of a pH of 10. When completely dissolved, the pH of the solution was checked with Hydrion paper and the solution was adjusted to a pH of 9 by the addition of a phosphoric acid solution at pH 6. The solution was then made approximately 0.33 per cent saline before injection by the addition of a suitably adjusted salt solution.

On May 19th rats numbered one and seven of Groups A and C, respectively, were injected with approximately 20 microcuries of radioactive phosphorus obtained from the Atomic Energy Commission, and made into a solution of disodium monohydrogen phosphate. The dilution was made by the Department of Chemistry in charge of that particular phase of isotope research. The injections were made subcutaneously as were the injections of the growth hormone. Safety precautions suggested by the Atomic Energy Commission were followed.

The animals were sacrificed by chloroforming them. The legs
of the rats were removed and cooked in boiling water for a few minutes, thus facilitating the removal of flesh from the bones. The femora and the humeri were the only bones saved for further use. Dehydration by a single immersion in a 50 per cent alcohol solution was all that was necessary before imbedding the bones.

The femora and the humeri were imbedded in Ward's Bioplastic. This was accomplished by first preparing a mixture of 25 cubic centimeters of plastic and six drops of catalyst. This mixture was poured into a small rectangular glass dish. Air bubbles were removed from the mixture by placing the glass dish containing the plastic in a bell-jar vacuum system. The plastic was caused to set slightly by placing it for a few minutes under an ultraviolet sunlamp. The bones were then placed on the plastic in such a fashion that it was possible to make cross-sectional cuts of the left bones and sagittal sections of the right ones. A second layer of the plastic-catalyst mixture was placed on the first layer, after which the air bubbles were again removed, and the preparations were then placed under the sunlamp. If the sunlamp was left on for about five hours and the plastic allowed to cool to room temperature, the plastic was ready to be cut and sanded. This procedure permitted the omission of heating the preparation at 37° C. during the curing process which ordinarily takes about 12 hours. Before the bones were sectioned, excess plastic was sawed and sanded away leaving a thin shell around the bone and a portion extending beyond in the form of a block to permit the imbedded bone to be clamped in the holder of the
specially adapted microtome.

It is impossible to cut bone sections with the ordinary knife-edge microtome without decalcifying the bone. Decalcification was impossible to use in studies dealing with radioactive salts of phosphorus. Also, older methods of bone sectioning would prove inadequate to the task of producing enough sections for a thorough study of the problem at hand. Axelrod (1943) states that in order to obtain optimum resolution the sections should be as thin as possible. An ordinary microtome was converted for the purposes of the experiment as described by Roofe, Hoecker, and Vorhees (1949), and Kaufman (1950). As the block containing the imbedded bone moved downward over the saw, a thin section of the block was sawed off.

The sections were washed in a very dilute solution of hydrochloric acid to remove any radioactive dust particles which might have been smeared over the bone section during the sectioning process. This washing in no way discernably affected the radioactive phosphorus which had been deposited in the bone.

The washed sections were next immersed in a solution of acetone and placed at one end of celluloid strips which had been cut to a size of one inch by three inches. Two, and sometimes three sections, were placed in this manner on each of the celluloid strips. The acetone dissolved some of the plastic which remained around the bone section and on contact with the celluloid dissolved a slight amount of the latter. The subsequent evaporation of the acetone left the bone section quite firmly
attached to the celluloid strip. This method proved to be very satisfactory as it allowed rapidity of manipulation and did not leave air bubbles between the bone section and the celluloid sheet as had been true for cement used in the early part of the experiment.

The bone sections, firmly attached to the celluloid sheets, were placed in contact with the emulsion of the photographic film. The film was cut to the same size as the celluloid. The ends of the film strip and the celluloid strip were then cemented together using Carter's airplane cement. This procedure proved to be very satisfactory. The development of the film after exposure, without immersion of the celluloid and the bone sections thereon, caused some strain on this cemented joint which, however, held sufficiently.

Evans (1948) pointed out that an autograph becomes more diffuse the greater the thickness of the tissue and the emulsion. Likewise, it becomes more diffuse the greater the distance between the tissue and the emulsion. Therefore, it was essential to hold the tissue firmly against the emulsion during exposure. The bone sections were pressed into close contact with the emulsion during the exposure. This close contact was obtained by placing the film-bone-celluloid strips between pieces of one-fourth inch plastic sheets in a cigar box. A few of these layers were put into each box. On the top of these layers, ingots of lead weighing approximately four pounds were used as weights. The cigar boxes were closed and made light-tight by sealing with
gummed tape.

Several types of film were tested in the early experimental work. Of the photographic emulsions tested, Eastman Kodak Portrait Panchromatic Film was found to give the best resolution. The small grain of this emulsion gave a very high resolution to the radioautograph. After an exposure of approximately 13 days, the film was developed by ordinary processes using Kodak D-50 developer and Kodak F-5 fixing solution. Care was taken to avoid immersing the bone sections in the developing solutions.

Photomicrographs were then made of both the radioautograph and the bone section. This procedure allowed a more detailed study of the two and made it possible to determine the more exact localization of the radioactive phosphorus.

EXPERIMENTAL RESULTS

With the injection of only small amounts (one, two, and four rat units per day per rat) of growth hormone, the injected rats show a slightly steeper slope in the growth curve. Upon increase of the dosage rate to 20 rat units per day per rat on the sixty-fifth day, the rate of growth increased as shown by the increased slope of the growth curves of the injected rats. The growth of the normal rats gradually leveled off throughout the experiment. This increased difference of the slope of the growth curves is especially noticeable when making a comparison of the growth curves of the injected female rats and that of the
normal females.

The animals considered in this experiment were sacrificed 19 hours after injection of the radioactive phosphorus.

The radioautographs of the bone sections of the rat injected with growth hormone (No. 1) were decidedly different from those of the noninjected rat (No. 7).

The photomicrograph of the radioautograph made of a sagittal section of the proximal end of the right femur of rat No. 1, sacrificed 19 hours after the phosphorus injection, shows a higher concentration of radiophosphorus in the epiphyseal line and in the diaphysis as compared with that of the epiphysis. The photomicrograph of the radioautograph of a similar bone section of rat No. 7, sacrificed 19 hours after injection, shows that in the uninjected rat the phosphorus was more evenly distributed throughout the epiphysis and the diaphysis with apparently equal amounts of the radio isotope in each of these areas. There was no concentration of the radioactive phosphorus at the epiphyseal line (Plate I).

Comparable results were noted in bone sections of the other rats.

DISCUSSION

Since growth hormone is known to accelerate growth, it is to be expected that the processes responsible for the gross manifestations of growth, the elongation of the bones, will be
EXPLANATION OF PLATE I

Positives of photomicrographs of bone sections and radioautographs.

A. A sagittal bone section of the proximal end of the right femur of rat No. 1 (growth hormone injected).

B. The radioautograph of the bone section shown in (A). Nineteen hours after injection of the radioactive phosphorus.

C. A sagittal bone section of the proximal end of the right femur of rat No. 7 (non-hormone injected).

D. The radioautograph of the bone section shown in (C). Nineteen hours after injection of the radioactive phosphorus.
best suited to demonstrate the activity of the hormone. In addition, since bone deposition involves the utilization of phosphates, the presence of increased amounts of radiophosphorus at the sites of deposition will be a criterion of such activity. This is verified by the results of the experiment.

The greater difference between the growth curves of the injected and noninjected females as compared to the difference between the growth curves of the injected and noninjected males may be due in part to the fact that normal female rats mature and reach the "plateau" sooner. The distinct difference in the slopes of the curves of rats injected with growth hormone and those not injected indicate that the injections of the growth hormone accelerated growth in general and that of the long bones which were studied, in particular.

The radioautographs extend and confirm what is known of bone phosphorus metabolism. Previous work (Hahn, Hevesy, and Lundgaard, 1937; Manly and Bate, 1939) has shown that phosphorus is first stored in the epiphysis and in the endosteum. This phosphorus is then remobilized and deposited in the epiphyseal line, the diaphysis, and periosteally during bone growth.

In the control (noninjected) rats, the rate of bone growth is comparatively slow as evidenced by a comparison of photomicrographs of the radioautograph D on Plate I with B and D on Plate II. Radioautographs D of Plate I and B of Plate II are of control rats sacrificed 19 hours after a single injection of radioactive phosphorus, while D of Plate II is of a rat sacri-
EXPLANATION OF PLATE II

Positives of photomicrographs of bone sections and radioautographs.

A. A cross section of the proximal half of the diaphysis of the left humerus of rat No. 7 (no growth hormone injected).

B. The radioautograph of the bone section shown in (A). Nineteen hours after injection of the radioactive phosphorus.

C. A cross section of the distal half of the diaphysis of the left femur of rat No. 8 (no growth hormone injected).

D. The radioautograph of the bone section shown in (C). Eighty hours after injection of the radioactive phosphorus.
ficed 30 hours after the injection. These radioautographs show that, in general, little mobilization in bone has taken place in the normal rat within a period of 30 hours.

Comparison of the radioautographs of the growth hormone injected rats (B) of Plate I with that of the noninjected rats (D) of Plate I shows that in growth accelerated rats mobilization of phosphorus had taken place within 19 hours after injection, and the phosphorus begins to appear in the region of the epiphyseal disc and under the periosteum. In other words, in control rats the phosphorus appears either diffused throughout the metaphysis and epiphysis or in some cases somewhat concentrated in the region of the endosteum. In rats whose growth has been accelerated by growth hormone, however, the distribution of the phosphorus appearing concentrated under the periosteum and in the epiphyseal disc, indicates a mobilization of the phosphorus from the sites it occupied in the control rats to the regions of accelerated bone growth.

In the study in which the rats were sacrificed 30 hours after injection, Kaufman (1950) found that the process of mobilization of phosphorus in growth hormone injected rats continued, as was indicated by a very marked area of the phosphorus under the periosteum.

Marx and Reinhardt (1942) using radioactive strontium found no difference in the strontium of bones in growth hormone injected rats as compared with those not receiving hormone. Since the radioactivity of the two piles of ground up bone containing
strontium was compared, it measured simply the total amount of strontium present in the bone itself, and did not show any differential distribution of the strontium. The present study clearly demonstrates that the distribution of radiophosphorus is quite different in bones of injected rats as compared to that of noninjected rats.

The results of this experiment indicate that growth hormone accelerates the mobilization of the bone phosphorus, and that the greatest activity is at the sites where new bone is formed, namely along the epiphyseal plate in young animals and under the periosteum in the young animals when growth is accelerated by injections of growth hormone of the anterior lobe of the pituitary gland.

SUMMARY

1. An experiment was performed using white rats (Rattus norvegicus albinus) to determine the effect of growth hormone on the mobilization of bone phosphorus.

2. The rats were divided into four groups. (Group A, injected with radioactive phosphorus and growth hormone; Group B, injected with growth hormone; Group C, injected with radioactive phosphorus; Group D, control).

3. Growth hormone was injected daily except Sundays for a period of 86 days.

4. The rats studied were sacrificed 19 hours after sub-
5. Cross sections and sagittal sections were made of the femora and humeri.

6. Radioautographs were made of these bone sections.

7. Photomicrographs of the bone sections and of the radioautographs indicate that within 19 hours after injection of the radioactive phosphorus growth hormone begins to accelerate the remobilization of the phosphorus from the epiphysis and endosteum to the epiphyseal line, the diaphysis, and the subperiosteal bone.
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